

Rachhpal S. Kahlon *Editor*

Pseudomonas: Molecular and Applied Biology



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Fondly dedicated to Iqbal Kahlon

Preface

In the early 1970s, when I joined the bandwagon of *Pseudomonas* as a postdoc at the Technical University of Denmark, Copenhagen, *Pseudomonas* was considered as a vaguely defined genus comprising soil-borne saprophytes, at times associated with wound infection. However, with the developments in molecular biology, genetic engineering, PCR, DNA, and genome sequencing supported by technological developments and computer-based databases, *Pseudomonas* has attained the position of a model organism for in silico modeling and systems biology where large chunks of unwanted DNA segment can be replaced by desired ones. Presently, it is an important organism for biotechnological production of novel chemicals and pharmaceuticals as well as for scientific pursuits for exploring new areas of its vast activities.

This volume was planned with a view to provide concise and comprehensive information to young graduates, teachers, and research workers to visualize the potential of this important and diverse group.

The first chapter on Phylogeny and Molecular Taxonomy has been contributed by distinguished scientists, Elena Garcia-Valdes and Jorge Lalucat, who discuss how the taxonomy of *Pseudomonas* has undergone a change and the members that were not true *Pseudomonas* were assigned to other subclasses of Proteobacteria. *Pseudomonas aeruginosa* an opportunistic pathogen is notorious for hospital-acquired infection, multiple drug resistance, and a cause of CF of lungs. Quorum sensing and the mechanism of pathogenesis and virulence have been discussed. Two chapters on cell envelope and metabolic network highlight the structure and function of cell envelope, particularly the outer membrane and the vast metabolic potential of *Pseudomonas*. Mono- and di-oxygenases are the key enzymes for degradation of recalcitrant molecules, and a chapter on these has been included. One chapter is dedicated to genome and comparative genomics of important species. Also a chapter on “in silico” modeling and use of bioinformatics and databases has been included. Two chapters on applied biology elaborate on commercial exploitation for production of novel molecules and biopolymers. The other highlights their role in degradation of organic chemical pollutants and bioremediation. The last two deal with *Pseudomonas*–plant interactions: one exclusively deals with plant pathogenesis by *Pseudomonas syringae* and the other elaborates their role on biocontrol of plant diseases and direct and indirect plant growth promotion.

I would like to place on record my appreciation and thanks to Alena and Jorge who were the first to accept my request to contribute a chapter on phylogeny and taxonomy. This acted as a catalyst and was a source of encouragement. I am equally grateful to Drs Levin, Parveen Sharma, Nagina, Swaranjit, Prince Sharma, and Saini and their colleagues for their outstanding contributions and making this volume possible.

I owe a great deal to my family, particularly my wife, Sukhdeep, for her all-round support and help. I do hope to compensate my lovely grandchildren Abhay and Amie by giving them more time now.

Financial support by way of grant from the Department of Science and Technology, Government of India, New Delhi, is acknowledged with thanks. I am grateful to Principal, SCD Govt College, Ludhiana, and Prof Dr B M Sarwar, Head, Dept of Botany, for implementation and smooth functioning of the project at their esteemed institute.

Lastly, I am grateful to my mentors, students, friends, and relatives who have been part of this beautiful journey. Timely help of Mr. Gurdeep Singh for computer typing is acknowledged with thanks.

Ludhiana, Punjab, India
December 14, 2015

Rachhpal S. Kahlon

Contents

1	<i>Pseudomonas</i>: Molecular Phylogeny and Current Taxonomy	1
	Elena García-Valdés and Jorge Lalucat	
2	Cell Envelope: Molecular Architecture and Function	25
	Rachhpal S. Kahlon	
3	<i>Pseudomonas</i>: The Versatile and Adaptive Metabolic Network . . .	81
	Partap Bir Singh, Harvinder Singh Saini, and Rachhpal S. Kahlon	
4	<i>Pseudomonas</i>: Genome and Comparative Genomics	127
	Rachhpal S. Kahlon	
5	<i>Pseudomonas</i> Oxygenases: Nature and Function	193
	Abha Shukla, Brijdeep Singh, Swaranjit Singh Cameotra, and Rachhpal S. Kahlon	
6	Quorum Sensing in <i>Pseudomonas aeruginosa</i>: Mechanism and Regulation of Virulence	231
	Sajal Sarabhai, Amanjot Kaur, Neena Capalash, and Prince Sharma	
7	<i>In Silico</i> Comparative Analysis of Type VI Secretion Systems in <i>Pseudomonas putida</i> LS46	257
	Parveen Kumar Sharma, Jilagamazhi Fu, Richard Sparling, and David Bernard Levin	
8	<i>Pseudomonas</i> for Industrial Biotechnology	281
	Rachhpal S. Kahlon	
9	Biodegradation and Bioremediation of Organic Chemical Pollutants by <i>Pseudomonas</i>	343
	Rachhpal S. Kahlon	

10	<i>Pseudomonas</i>-Plant Interactions I: Plant Growth Promotion and Defense-Mediated Mechanisms	419
	Hammad Khan, Nagina Parmar, and Rachhpal S. Kahlon	
11	<i>Pseudomonas</i>-Plant Interactions II: Biology and Pathogenesis of <i>Pseudomonas syringae</i>	469
	Rachhpal S. Kahlon	

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About the Editor

Rachhpal S. Kahlon holds a Ph.D. in Microbiology from CCS Haryana Agricultural University, Hisar, India, and Postdoctorate experience at Institute of Microbiology, Technical University of Denmark, Copenhagen, where he started working on *Pseudomonas*. After his return to India in 1976, he joined the Punjab Agricultural University, Ludhiana, and held the position of Professor cum Head, Department of Microbiology, and was Director (Biotechnology) Punjab State Council for Science and Technology, Government of Punjab, Chandigarh. His area of specialization has been *Pseudomonas* Genetics and Bioremediation and has successfully completed a number of research projects sanctioned by different departments of Government of India. He has over hundred publications in this area and has successfully guided 20 Master's and 15 Doctorate students. His work is well accepted and recognized and he has to his credit the first ever report on NIC, the nicotine-nicotinic acid degradative plasmid in *Pseudomonas convexa*.

Pseudomonas: Molecular Phylogeny and Current Taxonomy

1

Elena García-Valdés and Jorge Lalucat

Contents

1.1	The Genus <i>Pseudomonas</i> : Introduction and Historical Perspective	2
1.2	Housekeeping Genes Used in Molecular Phylogeny	5
1.3	Multilocus Sequence Analysis (MLSA) and Phylogeny	7
1.4	Main Characteristics of the <i>Pseudomonas</i> Phylogenetic Groups	9
1.5	Species Delineation in the Genus <i>Pseudomonas</i>	11
1.6	Digital Genome Analysis in <i>Pseudomonas</i> Taxonomy	12
1.6.1	ANI: Average Nucleotide Identity	12
1.6.2	Percentage of Conserved DNA	13
1.6.3	Digital DNA–DNA Hybridization for Microbial Species Delineation by Means of Genome-to-Genome Sequence Comparison (GGD)	13
1.6.4	Universal Single-Copy Marker Genes (MGs)	13
1.7	Molecular Identification and Molecular Typing Methods	15
1.7.1	Whole-Cell Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (WC-MALDI-TOF MS)	15
1.7.2	<i>Pseudomonas</i> Selective <i>rpoD</i> Gene PCR Primers	16
1.7.3	Molecular Typing	17
1.8	Detection of <i>Pseudomonas</i> in Environmental DNA by <i>rpoD</i> Selective Primers	18
1.9	Future Prospects	19
	References	20

Abstract

The actual backbone of *Pseudomonas* taxonomy relies on the molecular phylogenetic relationships among the species. The 16S rDNA permits the adscription of a strain in the genus, but its resolution at intrageneric level is low in *Pseudomonas* genus. Other housekeeping genes have been proposed for the species differentiation in the genus (*gyrB*, *rpoD*, *rpoB*, etc.). Individual and

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consensus analysis of housekeeping genes (multilocus sequence analysis, MLSA) allows the differentiation of the *Pseudomonas fluorescens* lineage (divided into six groups) and the *Pseudomonas aeruginosa* lineage (divided into four groups). MLSA and experimental DNA–DNA hybridization (DDH) continue to be the main molecular criteria for prokaryotic species delineation, but whole-genome sequences are providing valuable information on the evolutionary and taxonomic relationships in bacteriology. Very useful methodologies are the digital calculations of the average nucleotide identity (ANI), the percentage of conserved DNA, genome-to-genome sequence comparison (GGD), or the analysis of universal single-copy marker genes (MGs). Molecular identification and typing methods based on MLSA or single genes analysis (as *rpoD*) are reliable for the species differentiation (and for strains differentiation within a species), but the recently applied whole-cell matrix-assisted laser desorption/ionization time-of-flight (WC-MALDI-TOF) mass spectrometry (MS) has gained popularity, and it is also a useful tool for the phenotypic characterization and identification of *Pseudomonas* strains. The use of *Pseudomonas* selective PCR primers allows the detection of *Pseudomonas* in environmental samples and demonstrates the high diversity of the genus and that the total species diversity of the genus has not been fully described yet.

1.1 The Genus *Pseudomonas*: Introduction and Historical Perspective

The *Pseudomonas* genus description has changed substantially since the simple description given by Schroeter (1872) of the *Bacterium aeruginosum* and the later proposal of Migula (1895) for the genus *Pseudomonas*. Taxonomic characteristics of the genus have varied along the almost 150 years since the first definition, in accordance with the taxonomic criteria and tools available at each moment. The simple morphological description of Migula (cells with polar organs of motility) was more precise when he proposed *Pseudomonas pyocyanea* as the type species of the genus (Migula 1895), later renamed *Pseudomonas aeruginosa*. The methods introduced by den Dooren de Jong (1926) in Beijerinck's laboratory in Delft for extensive phenotypic (morphological, physiological, biochemical) characterization of strains were applied by R. Y. Stanier, N. J. Palleroni, and M. Doudoroff to *Pseudomonas* strains. The results were published in a "citation classic" article in the Journal of General Microbiology (Stanier et al. 1966). The study of phenotypic properties of many strains representative of species of the genus was also used for the definition of species groups. Later, Sneath et al. (1981) analyzed by numerical taxonomy the phenotypic records published and found good agreement with the groupings delineated previously within the genus.

Studies of Palleroni et al. (1973) demonstrated that the level of rRNA homology in rRNA/DNA hybridization experiments allowed the classification of

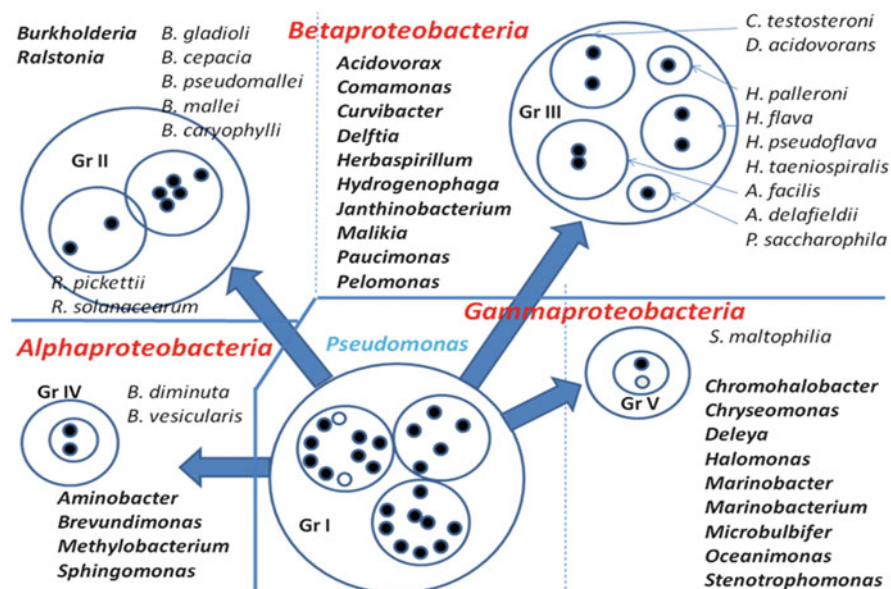


Fig. 1.1 Actual taxonomic distribution of former *Pseudomonas* in the five DNA–RNA homology groups proposed by Palleroni et al. (1973)

Pseudomonas species into five groups. These results were confirmed by Moore et al. (1996) and Anzai et al. (2000) in phylogenetic studies based on the 16S rRNA sequence and provoked the splitting of species in the genus *Pseudomonas* in at least 25 genera in the actual taxonomy. Some species were transferred to four genera already existing or newly created in the classes *Alphaproteobacteria*, 12 genera of the *Betaproteobacteria*, and 13 genera in the *Gammaproteobacteria*, as depicted in Fig. 1.1. Many of these genera are distant to the true members of the genus in rRNA/DNA homology group I, which is considered to constitute the genus *Pseudomonas* sensu stricto and includes *P. aeruginosa*, the genus type species.

Phenotypic analyses in strains of the genus were completed by comprehensive chemotaxonomic investigations. Results showed that each of the *Pseudomonas* groups detected by Palleroni and collaborators could be easily discriminated. Studies of the fatty acid composition of *Pseudomonas* species (Ikemoto et al. 1978) revealed that the straight-chain saturated fatty acid C16:0 and the straight-chain unsaturated fatty acids C16:1 and C18:1 were the most abundant in *Pseudomonas* species. The determination of polyamine and quinone composition is a rapid chemotaxonomic identification tool. Putrescine (Q9) is the main component of all members of the genus *Pseudomonas* (Busse and Auling 1988).

Pseudomonas species are common in natural habitats due to their high capacity for the mineralization of organic matter. Strains of *Pseudomonas* species can be cultured on simple mineral medium with a single organic compound, without the need of nutritional supplements. Strains can be enriched and isolated from many sources, water, soil, plants, animals, aliments, etc. Some of them are pathogenic for

plants and animals. Many species have been published for newly isolated strains, and the genus has been along the years a good example for the development and validation of new taxonomic tools in bacteriology. As a result, the number of species included in the genus has changed according to the taxonomic tools available. In the 7th edition of the Bergey's Manual of Determinative Bacteriology (1957), 149 species were described; in the 8th edition (1974), 235 species (29 in four sections and 206 in addenda); in the Approved Lists of Bacterial Names, (1980) 87 species and 31 as *Incertae sedis*; 23 species and 64 additional species in the 9th edition of the Manual (1994); in the Bergey's Manual of Systematic Bacteriology 1st edition (1984), 82 species are described in three sections; and in the Bergey's Manual of Systematic Bacteriology 2nd edition (2005), 53 species and 8 species with uncertain phylogenetic position are described. The number of species is increasing continuously and other species have been transferred to other genera. At this moment, 213 *Pseudomonas* species are cited in the List of Prokaryotic Names with Standing in Nomenclature (Parte 2014), but only 147 *Pseudomonas* species are accepted in the actual taxonomy. The number of species recognized each year since the first *Pseudomonas* description is presented in Fig. 1.2.

As described by Palleroni in the 2nd edition of the Bergey's Manual of Systematic Bacteriology (2005), the genus *Pseudomonas* is characterized by: "Straight or slightly curved rods but not helical. Most of the species do not accumulate granules of polyhydroxybutyrate, but accumulation of polyhydroxyalkanoates of monomer

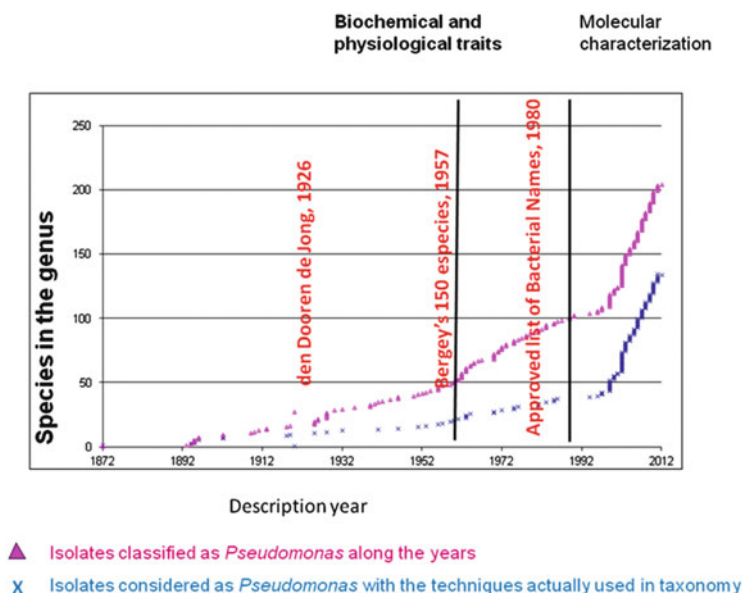


Fig. 1.2 Major changes in the methods used and species numbers in *Pseudomonas* taxonomy. In the Approved list of Bacterial Names (1980), 2212 bacterial species were recognized; 96 of them were *Pseudomonas* species

lengths higher than C₄ may occur when growing on alkanes or gluconate. Motile by one or several polar flagella. Aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor, in some cases nitrate can be used as an alternative electron acceptor. Oxidase positive or negative. Chemoorganotrophic. Strains of the species include in their composition the hydroxylated fatty acids C10:0 3OH and C12:0 and C12:0 2OH and ubiquinone Q9.” We can add to the definition the need of close phylogenetic relationships in DNA sequences among species in the genus and also that the species should be monophyletic with *P. aeruginosa*, the type species in the genus.

The most closely related bacteria to the genus *Pseudomonas* include the species of the aerobic, free-living, nitrogen-fixing *Azotobacter*–*Azomonas* complex and cellulolytic species of the genus *Cellvibrio* (some strains were previously known as “*Pseudomonas fluorescens* subsp. *cellulosa*”).

It is generally recognized in bacterial taxonomy that a polyphasic approach is the best way to classify bacteria (Vandamme et al. 1996). Polyphasic taxonomy is aiming at the integration of phenotypic, genotypic, and phylogenetic information for the classification and identification of bacteria. We will introduce in the next sections different kinds of molecular approaches that are useful for *Pseudomonas* taxonomy. Most of them have been developed in the last 10 years.

1.2 Housekeeping Genes Used in Molecular Phylogeny

Several genes have been used to delineate the phylogenetic status of species in the genus *Pseudomonas*. The 16S rDNA as a universal marker permits the adscription of a strain in the genus and allows comparisons between very divergent bacteria (Santos and Ochman 2004). Moore et al. (1996) and Anzai et al. (2000) published their studies on the phylogeny of *Pseudomonas* based on the analysis of the 16S rDNA, although it was demonstrated later that its resolution at intragenic level was low in *Pseudomonas* genus. Therefore, other housekeeping genes were proposed for the species differentiation in the genus. Yamamoto and collaborators incorporated the use of the *gyrB* and *rpoD* genes, and 23 taxa were analyzed phylogenetically (Yamamoto et al. 2000). The *gyrB* gene encodes the beta-subunit of the gyrase (EC 5.99.1.3), responsible for the negative super coiling of the DNA, and *rpoD* is the gene encoding the sigma 70 subunit of the RNA polymerase (EC 2.7.7.6). Both genes, *gyrB* and *rpoD*, have been used initially for the phylogenetic characterization of *Pseudomonas putida* strains, and later for 31 species of the *Pseudomonas* genus, establishing in it different complexes of species (Yamamoto and Harayama 1998; Yamamoto et al. 2000).

The *rpoB* gene, encoding the beta-subunit of the RNA polymerase (EC 2.7.7.6), has been postulated as a good candidate for phylogenetic analysis and identification of bacteria for clinical microbiologists (Adékambi et al. 2009). In the *Pseudomonas* genus, this gene has been used by Tayeb and collaborators (2005) but also in some other organisms, like *Brevundimonas*, *Ralstonia*, *Comamonas*, or *Burkholderia* (Tayeb et al. 2008), many of them former members of the genus *Pseudomonas* (*sensu lato*).

Other useful gene sequences have been tested by several authors, although the number of species studied was limited: the internally transcribed 16S–23S spacer ITS1 region (Guasp et al. 2000); *gacA* (de Souza et al. 2003); *oprI* and *oprL* (Matthijs et al. 2013); *atpD*, *carA*, and *recA* (Hilario et al. 2004); *FlaA*, *RpoS*, and *RpoH* (Kiil et al. 2008); and *rrs*, *dsbA*, *gyrB*, *rpoD*, *fdxA*, *recA*, *rpoB*, *fusA*, *rpsL*, and *rpsG* (Frapolli et al. 2007). Kiewitz and Tümmler (2000) studied six genes (*oriC*, *citS*, *ampC*, *oprI*, *fliC*, *pilA*) for *P. aeruginosa* strains.

An important issue in the decision of the gene sequences to be used in phylogenetic studies is their relative discriminatory power and the possible correlation of the similarities among strains using different genes. Four housekeeping genes were selected by Mulet et al. (2010) for a multigenic phylogenetic analysis of 107 type strains of the *Pseudomonas* genus: 16S rRNA, *gyrB*, *rpoB*, and *rpoD* genes. The four genes were compared in order to select the most discriminating gene. For each single gene, a matrix of the phylogenetic distances between the 107 type strains was constructed, and the distances of pairs of strains (5671 values) were plotted. Pair-wise comparisons demonstrated that the *rpoD* distances correlated with R^2 values of 0.64, 0.75, and 0.69 for the 16S rRNA, *gyrB*, and *rpoB* genes, respectively. The discriminatory power of each gene was calculated as the ratio between the *rpoD* slope and the slopes of the other genes: *rpoD*/16S rDNA (eight times), *rpoD*/*rpoB* (three times), and *rpoD*/*gyrB* (two times). The more discriminating gene analyzed was *rpoD*, followed by *gyrB*, *rpoB*, and the 16S rRNA gene. The range and average distances for each gene are shown in Fig. 1.3. A similar study was performed by

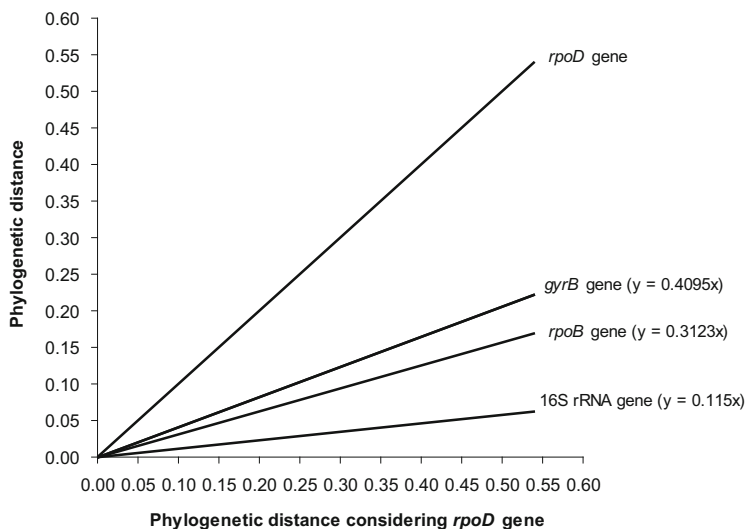


Fig. 1.3 Least square tendency lines obtained for the phylogenetic distance between 107 type strain *Pseudomonas* for three different genes (16S rDNA, *gyrB*, and *rpoB*) with respect to the *rpoD* gene. The slope is indicated in each case. The lines have been vertically shifted for the sake of clarity. The correlation coefficient R^2 is 0.6401 to 16S rDNA, 0.7501 to *gyrB*, and 0.686 to *rpoB* (Mulet et al. 2010)

Scotta et al. (2013) with a collection of 229 *P. stutzeri* strains. The *gyrB* and *rpoD* genes showed a similar number of polymorphic sites (42.5 and 42.4 %, respectively), but the *rpoD* gene exhibited higher number of informative sites (38.8 %) than *gyrB* gene (37.1 %). The evolutionary pressure upon *rpoD* and *gyrB* genes was quantified through the dN/dS ratio. This value was lower than one in both protein-coding genes indicating that they are under purifying selection pressure.

Similarly, three other genes (*atpD*, *carA*, *recA*) from 13 type strains (Hilario et al. 2004) were compared, and the *rpoD* was also the most discriminating gene (Mulet et al. 2010). The genetic diversity of *oprI* and *oprL* sequences was also compared with *rpoD* sequences (Matthijs et al. 2013). The discriminatory power of the *rpoD* gene was three times higher than *oprI* gene and two times higher than *oprL* gene.

1.3 Multilocus Sequence Analysis (MLSA) and Phylogeny

The ad hoc committee for the reevaluation of the species definition in bacteriology (Stackebrandt et al. 2002) has recommended the use of several housekeeping genes for phylogenetic reconstruction in bacterial taxonomy. As mentioned before, Yamamoto et al. (2000) were the first investigators to include the analysis of several housekeeping genes in the phylogeny of *Pseudomonas*. Later, Hilario and collaborators (2004) incorporated sequences of *atpD*, *carA*, and *recA* genes into the analysis of 13 type strains of *Pseudomonas* (together with other reference strains). The phylogenetic relationships between *Pseudomonas* species based on DNA sequencing of representative genes were not routine until the present decade, and only few considered the combined phylogenetic analysis of several genes in some species. In the last years, besides the mandatory 16S rRNA gene sequence in descriptions of new species, most of them included *gyrB* and *rpoD* or *rpoB* gene sequences.

The phylogenetic trees derived from the analysis of individual housekeeping genes show similar topologies, maintaining the groupings and branching order in most cases. To infer a more robust phylogeny of the genus, several genes were used in a combined analysis. Partial sequences of the 16S rRNA, *gyrB*, *rpoB*, and *rpoD* genes of 107 *Pseudomonas* strains were analyzed by Mulet and collaborators (2010). This work demonstrated that the concatenated analysis of three genes (16S rRNA, *gyrB*, and *rpoD*) was enough for the phylogenetic analysis of the genus. The inclusion of *rpoB* may be necessary in some cases, but it does not improve the resolution in discriminating the type strains. Individual gene trees, as well as the concatenated sequences and a consensus analysis, allowed the discrimination of two lineages in the genus *Pseudomonas*, called: *P. fluorescens* lineage (Fig. 1.4a) and *P. aeruginosa* lineage (Fig. 1.4b). The bootstrap values of each complex branch of the individual, three, or four concatenated genes analyzed showed the robustness of the analysis. Mulet et al. in 2012 updated the previous work studying 138 *Pseudomonas* strains (135 *Pseudomonas* type strains, *P. alkylphenolica*, and 2 *P. chlororaphis* subspecies), including recently described

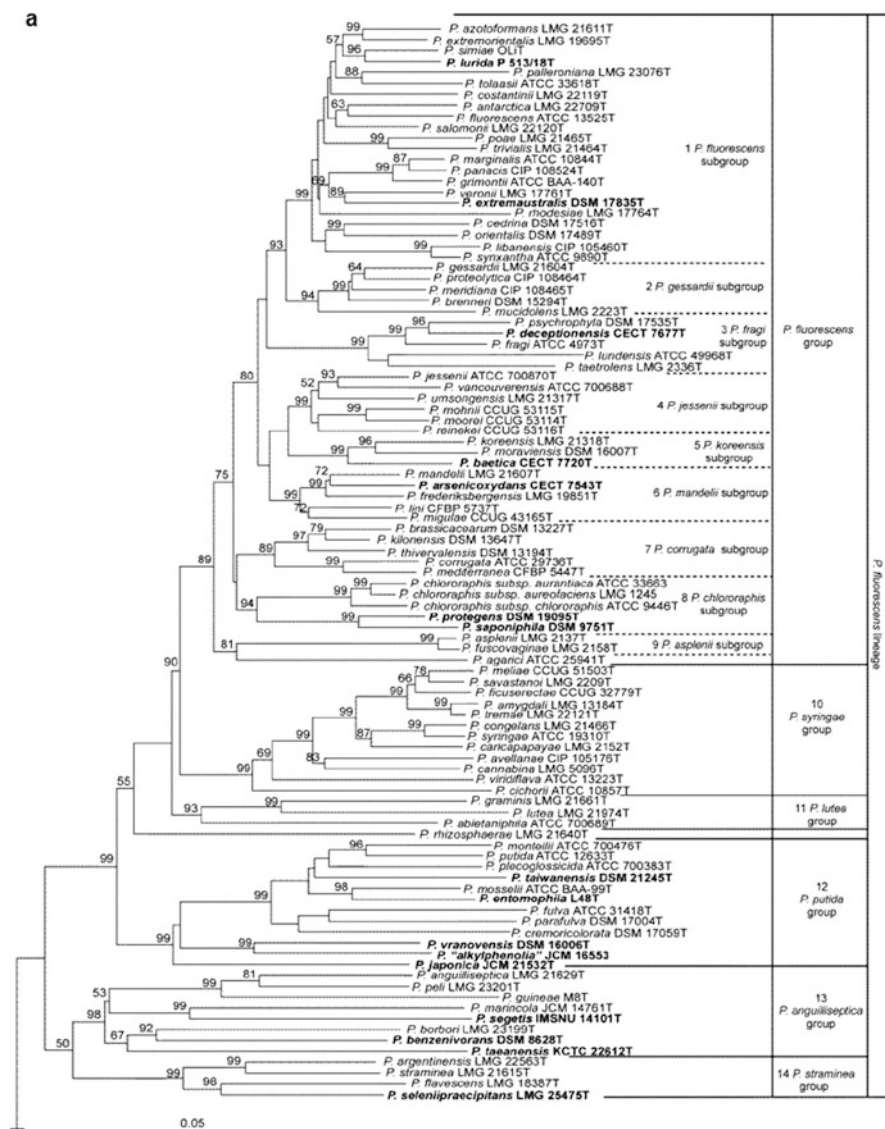


Fig. 1.4 Phylogenetic tree of 136 *Pseudomonas* type strains based on phylogenetic analysis of partial sequences of the 16S rRNA, gyrB, rpoB, and rpoD genes. The bar indicates sequence divergence. Distance matrix was calculated by the Jukes–Cantor method. Dendrogram was generated by neighbor joining. *Cellvibrio japonicus* Ueda107 was used as outgroup. Intrageneric groups (IG) or lineages, called Lineage *P. fluorescens* and Lineage *P. aeruginosa*, groups and subgroups have been marked (Mulet et al. 2010). (a) Phylogenetic branch of the *P. fluorescens* lineage, (b) phylogenetic branch of the *P. aeruginosa* lineage (Mulet et al. 2012). The species indicated in **bold** correspond to the most recent added species. The species in the **box** correspond to the new described group *P. pertucinogena*. Bootstrap values of more than 500 (from 1000 replicates) are indicated at the nodes

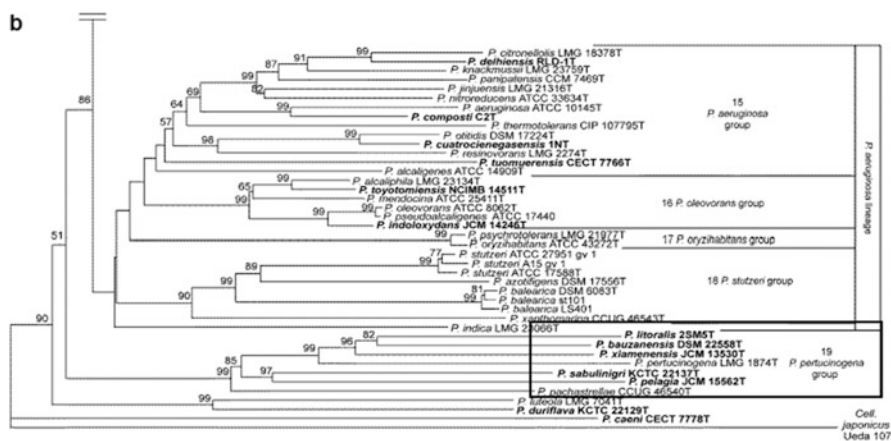


Fig. 1.4 (continued)

Pseudomonas species in order to reach a comprehensive view on the phylogenetic relationships of the species in the *Pseudomonas* genus (Mulet et al. 2012). The matrices constructed for the concatenated partial sequences of three genes (16S rRNA gene, *gyrB*, *rpoD*; 2870 nt) and four genes (16S rRNA gene, *gyrB*, *rpoB*, and *rpoD*; 3726 nt) were compared in a pair-wise manner to assess the correlation between them and the relative discriminatory power of both sets of genes. They were well correlated and almost equally discriminating (three genes vs. four genes, $y = 1.0252x$, $R^2 = 0.987$). *rpoD* was well correlated with all of them (three genes, $y = 0.3741x$, $R^2 = 0.9077$; four genes, $y = 0.3586x$, $R^2 = 0.8881$) and yielded the best resolution. Similar results were obtained in a study of *P. stutzeri* strains to determine their assignment to genomovar in the species *P. stutzeri* (Scotta et al. 2013).

1.4 Main Characteristics of the *Pseudomonas* Phylogenetic Groups

Two lineages were clearly differentiated. The first, *P. fluorescens* lineage, was divided into six groups (G), each one represented by the species *P. fluorescens* (56 species), *P. syringae* (12 species), *P. lutea* (three species), *P. putida* (12 species), *P. anguilliseptica* (eight species), and *P. straminea* (four species) (Fig. 1.4a). The *P. fluorescens* group was the most complex and included nine subgroups (SG) that were represented by the species *P. fluorescens*, *P. gessardi*, *P. fragi*, *P. mandelii*, *P. jesseni*, *P. koreensis*, *P. corrugata*, *P. chlororaphis*, and *P. asplenii*. The second lineage, of *P. aeruginosa*, was divided into four main groups, represented by the species *P. aeruginosa* (15 species), *P. oleovorans* (six species), *P. stutzeri* (four species), and *P. oryzae* (two species) (Fig. 1.4b).

P. agarici and *P. rhizosphaerae* were affiliated in the phylogenetic analysis within the lineage *P. fluorescens* and *P. indica* in the lineage *P. aeruginosa* but were independent of any group. Species of the *P. pertucinogena* group were independent of any lineage. Actually, it is a consolidated group in which several new species have been included. *P. luteola*, *P. caeni*, and *P. duriflava* should be considered outliers of the genus.

Most species are included in phylogenetic groups which are coincident with the classical phenotypic groups of *P. fluorescens*, *P. syringae*, *P. putida*, *P. stutzeri*, and *P. aeruginosa*, and the characteristics of each of them can be extended probably to the whole group.

Pseudomonas fluorescens is a common environmental bacterium frequently recovered from soil, water, and plant surfaces. Many strains of this species are used as biocontrol and plant growth-promoting agents, although strains associated with plant disease are also frequently found within this species complex.

Pseudomonas syringae is a foliar plant pathogen that causes a variety of blight, speck, and spot diseases in many important agricultural crops including tomato, soybeans, rice, and tobacco (Guttman et al. 2008). Over 67 different pathogenic varieties (pathovars) have been named within this group. As demonstrated by Parkinson et al. (2011), the pathovars can be also differentiated by sequencing the *rpoD* gene.

P. putida is a soil and plant-associated bacterium. It is of interest as an important biodegradative species that is capable of eliminating some of the most deadly and challenging environmental toxins (Wackett 2003).

P. stutzeri is defined as a soil bacterium with important biodegradative and bioremediation capabilities. It is able to colonize plant roots epiphytically. Due to the ability of nitrogen fixation by some strains in the species, it has been developed as bioinoculant to stimulate plant growth (Guttman et al. 2008; Silby et al. 2011). However, many strains are frequently isolated from clinical specimens (Scotta et al. 2013). Twenty-one genomovars (a provisional taxonomic status for genomic groups within a species that cannot be differentiated phenotypically; Ursing et al. 1995) have been already found in *P. stutzeri* (Cladera et al. 2004; Scotta et al. 2013). For a review of the species, see Lalucat et al. (2006).

P. aeruginosa is an opportunistic human pathogen mainly associated to cystic fibrosis although water is considered its primary habitat reservoir (Selezska et al. 2012). It can infect a wide range of organisms, including plants, nematodes, fruit flies, wax moth, zebrafish, and various mammals. In humans, *P. aeruginosa* is also responsible of chronic lung infections. It contributes to the burden of hospital-acquired infections, where it is the cause of respiratory and urinary tract infections (Silby et al. 2011 and the references therein).

1.5 Species Delineation in the Genus *Pseudomonas*

Bacterial species are considered to be groups of strains that are characterized by a certain degree of phenotypic consistency, by a significant degree (70 %) of DNA–DNA hybridization (DDH; Wayne et al. 1987) and over 98.7–99 % of 16S ribosomal RNA gene sequence similarity (Stackebrandt and Ebers 2006). Alternatively, a threshold of 97 % of similarity in the MLSA study of four housekeeping genes (16S RNA, *gyrB*, *rpoD*, and *rpoB* genes) has been proposed by Mulet et al. (2010, 2012) for the species differentiation in the genus *Pseudomonas* (Table 1.1).

Some species of the genus *Pseudomonas*, as *P. aeruginosa*, are taxonomically homogeneous and are relatively easy to differentiate and identify, but other species are much more difficult to identify. Moreover, some species are divided in intra-specific categories for their physiological and biochemical characteristics (*P. fluorescens* and *P. putida* in biovars), for their pathogenic properties against plants (*P. syringae* and *P. marginalis* in pathovars) and for their genomic differences (*P. stutzeri* genomovars). As an example of the molecular techniques applied to the species circumscription in the genus *Pseudomonas*, we can mention the study of 33 strains previously classified as *Pseudomonas putida* that were phylogenetically affiliated with their closest relatives in the genus by multilocus sequence analysis (Mulet et al. 2013). The results demonstrated that strains assigned to biovar A of the species were located in the *P. putida* group, though not all belonged to the species *P. putida*. Biovar B strains were scattered among six subgroups of the *Pseudomonas fluorescens* group and also within the *P. putida* group. The phylogenetic results show that isolates of biovars A and B are in distinct phylogenetic groups and are not monophyletic, and thus, these biotypes are not reliable taxonomic markers in the actual taxonomy.

Very useful databases in *Pseudomonas* taxonomy are freely available at the internet. Euzéby's List of Prokaryotic Names with Standing in Nomenclature

Table 1.1 Threshold values recommended for different methods in the *Pseudomonas* species assignation

Method for <i>Pseudomonas</i> species assignation	Percentage value	Reference
16S rRNA gene	98.7–99	Stackebrandt and Ebers (2006)
All species living tree (LTP)	98.7	Yarza et al. (2010)
<i>rpoD</i> gene	95–96	Mulet et al. (2010)
DNA–DNA hybridization	70	Wayne et al. (1987)
Multilocus sequencing analysis (MLSA)	97	Mulet et al. (2012)
Average nucleotide identity (ANiB)	95	Goris et al. (2007), Gomila et al. (2015)
Conserved DNA	69	Goris et al. (2007)
Genome-to-genome distance (GGD)	70	Auch et al. (2010), Gomila et al. (2015)
Universal marker genes (MGs)	96.5	Mende et al. (2013)

provides a comprehensive list of species that have been named *Pseudomonas*, and it is monthly updated with the new species proposed; The All-Species Living Tree project (<http://www.arb-silva.de/projects/living-tree/>) and the EzTaxon Biocloud (<http://www.ezbiocloud.net/eztaxon>) are curated databases of 16S rRNA sequences which greatly facilitate the species identifications; PseudoMLSA is a sequence-based database specialized in *Pseudomonas* strains (Bennasar et al. 2010).

1.6 Digital Genome Analysis in *Pseudomonas* Taxonomy

Although housekeeping gene sequences and experimental DDH continue to be considered the main molecular criteria for prokaryotic species delineation, much additional taxonomic information can be extracted from complete genome sequences (Coenye et al. 2005). Whole-genome sequences can provide valuable information on the evolutionary and taxonomic relationships in bacteriology. Already in 2005, Coenye and collaborators published an article entitled “Towards a prokaryotic genomic taxonomy” and presented an overview of approaches that were available to assess the taxonomic relationships between prokaryotic species based on complete genome sequences (Coenye et al. 2005). In the following section, we will describe three methodologies tested in our laboratory and useful in *Pseudomonas* taxonomy (Gomila et al. 2015). These genomic methods are delineated to substitute the experimental DDH by providing the possibility to create accumulative databases of whole-genome sequences.

1.6.1 ANI: Average Nucleotide Identity

Konstantinidis and Tiedje (2005) and Goris et al. (2007) proposed the ANI value for the species delineation in prokaryotes. The method consists in pair-wise, whole-genome sequence comparisons performed as follows: the genomic sequence from one of the genomes in a pair (“the query”) is cut into consecutive 1020 nt fragments. The 1020 nt fragments are then used to search against the whole-genomic sequence of the other genome in the pair (“the reference”) by using the BLASTN algorithm (Altschul et al. 1997). The ANI between the query genome and the reference genome is then calculated as the mean identity of all BLASTN matches that showed more than 30 % overall sequence identity over an alignable region of at least 70 % of their length. ANI of common genes among two strains shows a strong linear correlation to DNA–DNA reassociation values determined experimentally, and the 70 % DDH standard corresponds to 95 ± 0.5 % ANI. Strains that show more than 95 % ANI should belong to the same species. Goris et al. (2007) concluded that ANI can accurately replace DDH values for strains for which genome sequences are available (Table 1.1).

1.6.2 Percentage of Conserved DNA

To calculate the percentage of conserved DNA between a query and a reference, only those BLASTN matches reaching values above a cutoff point of 90 % nucleotide sequence identity are considered, regardless of the extent of the alignable region. The lengths of the alignable regions for all such matches are summed, and the sum was divided by the total length of the genomic DNA of the query genome to provide a genome size-independent measurement of the percentage of the query's DNA that was conserved in the reference genome. The recommended cutoff point of 70 % DDH for species delineation corresponds to 95 % ANI and 69 % conserved DNA (Table 1.1).

The work package JSpecies is a user-friendly, biologist-oriented interface to calculate ANI and conserved DNA (Richter and Rosselló-Móra 2009). It is available at the internet in the web page: <http://www.imedea.uib.es/jspecies/about.html>.

1.6.3 Digital DNA–DNA Hybridization for Microbial Species Delineation by Means of Genome-to-Genome Sequence Comparison (GGD)

This bioinformatic method was also designed to replace the experimental DNA–DNA hybridization (Auch et al. 2010). Digitally derived genome-to-genome distances show a good correlation with 16S rRNA gene sequence distances and with the experimental DDH values. The method performs well with complete closed genomes but also with incomplete sequenced genomes (drafts). The principle is that two genomes are locally aligned using BLAST, which produce a set of high-scoring segment pairs (HSPs); the information in these HSPs is transformed in a single genome-to-genome distance value by the use of a specific distance formula that sets the species cutoff at 70 % similarity. The genome-to-genome distance calculator (GGDC) has been developed independently of the ANI. It is available at the internet site <http://ggdc.dsmz.de> (Table 1.1; Fig. 1.5).

1.6.4 Universal Single-Copy Marker Genes (MGs)

Mende et al. (2013) proposed a different method to infer the species circumscription in prokaryotes. A genome sequence is assigned to a known species by the SpecI software without considering phylogenetic algorithms. The method is freely available at the EMBO web page (<http://vm-lux.embl.de/~mende/specI/index.html>). This web server automatically extracts the 40 universal single-copy marker genes (MGs) (Ciccarelli et al. 2006; Sorek et al. 2007) from a given genome and performs distance calculations to a database of MGs from ca. 3500 genomes. The genome is assigned to a species if its MGs are on average more than 96.5 % identical to all genomes assigned to a species (Table 1.1). Figure 1.6 gives an example of the results obtained with *P. syringae* strains.

Genome-to-Genome Distances (GGD) dendrogram

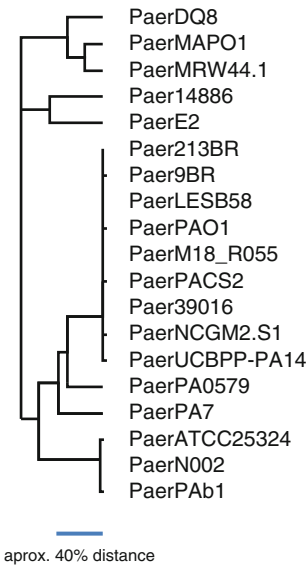


Fig. 1.5 Dendrogram of selected *P. aeruginosa* strains based on the genome-to-genome distances

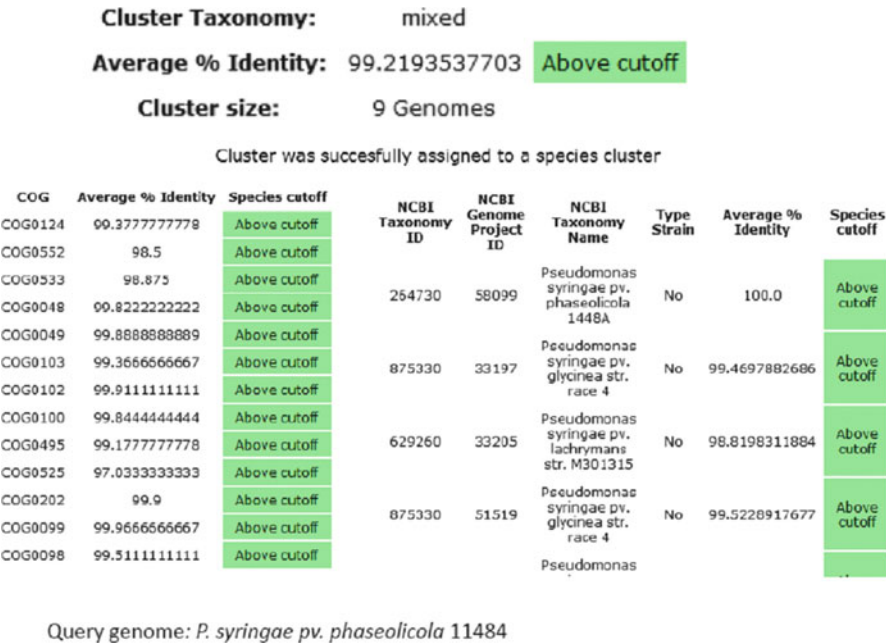


Fig. 1.6 Partial results of the species clustering of *P. syringae* pv *phaseolicola* 11484 with the SpecI program

1.7 Molecular Identification and Molecular Typing Methods

Although the taxonomy of *Pseudomonas* and associated identification methods have evolved with the available methodologies, the rapid and reliable identification of strains remains the most important task in any taxonomic study. Multilocus sequence analysis (MLSA) is actually the most accepted method for the phylogenetic assignation of *Pseudomonas* strains to their corresponding species. It has proven reliable for the species discrimination and strain identification in *Pseudomonas*. However, several phenotypic methods are currently available giving satisfactory results for the most commonly isolated species. Some methods rely on the physiological and biochemical properties of the strains, as in the Biolog, API, Multiscan, or Vitek systems that are used in routine identifications. Others, like FAME (fatty acid methyl ester analyses), are based on the chemotaxonomic analysis of the lipids extracted from whole cells. In any identification system, the accuracy depends on the quality of the database, and updated databases are essential for correct bacterial identification as the number of *Pseudomonas* species is increasing continuously. Identification methods are well described in Moore et al. (2006). A novel method, whole-cell matrix-assisted laser desorption/ionization time-of-flight (WC-MALDI-TOF) mass spectrometry (MS) analysis has been applied very recently to the identification of bacteria and is considered to be a fast and reliable method. WC-MALDI-TOF MS has been applied to the genus *Pseudomonas* as a complementary technique in the proposal of novel species, and the concordance with MLSA in the genus has been published recently in which the usefulness of the information obtained via both MLSA and WC-MALDI-TOF MS techniques was evaluated (Mulet et al. 2012).

1.7.1 Whole-Cell Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (WC-MALDI-TOF MS)

A total of 133 type strains of the recognized species and subspecies in the genus *Pseudomonas*, together with other representative strains, were analyzed using this new technique, which is fast and simple. The methodology using a whole-cell protocol is described in detail in Mulet et al. (2012). Cells were cultured on LB plates and colonies were picked for the analysis. The mass spectra were accumulated from 100 profiles, each from five nitrogen laser pulse cycles, by scanning the entire sample spot. The ions were accelerated with pulsed extraction at a voltage of 20 kV. The profiles of the peaks obtained for each species within a mass range from 3000 to 20,000 Da were analyzed and compared using the BGP database software available at the website <http://sourceforge.net/projects/bgp>. The percentage similarities of identical mass peaks were calculated and used to generate a dendrogram using the PermutMatrix software, applying an average-linkage method (UPGMA hierarchical clustering) and Pearson's distance correlation. The strains were also identified by comparing the resulting mass fingerprints with the SARAMIS (Spectral Archiving and Microbial Identification System, Release 3.36,

AnagnosTec, and RIPAC GmbH, Germany) database. The method has been also tested in our laboratory using a Bruker Autoflex mass spectrometer and the identification performed with the Biotyper database and an in-house database. All the species and subspecies were well discriminated in the WC-MALDI-TOF MS analysis. The similarities between species were lower than 60 %. Even when the 19 groups or subgroups established using the MLSA phylogenetic analysis could not be discriminated consistently using the WC-MALDI-TOF MS analysis, a good correspondence with the WC-MALDI-TOF MS dendrogram was found (82.9 % of the strains were grouped exactly by both methodologies). This good correspondence in the groupings indicated a strong phylogenetic signal in the mass spectral analysis. This result can be explained by the fact that the most important signals in the mass spectra correspond to ribosomal proteins and these proteins coevolved with the 16S rDNA, which has a strong weight in the MLSA technique.

For species-level differentiation and identification purposes in ecological and clinical microbial studies, the WC-MALDI-TOF MS approach can be a good method of choice because it is fast and accurate when the reference database is exhaustive (Scotta et al. 2013).

1.7.2 *Pseudomonas* Selective *rpoD* Gene PCR Primers

Several PCR primer sets have been developed for the selective amplification of *Pseudomonas* strains. The primers designed by Widmer et al. (1998) were based on highly specific sequences to the 16S rRNA gene producing an amplicon of approximately 990 bp. The major drawback of the method is the limited species resolution of the sequence. Alternatively, Locatelli et al. (2002) proposed the use of specific primers designed for the 16S and the 23S rDNA based on the high discriminatory power of the ITS1 region. The targeted segment is relevant for identification at the species, as well as at the intraspecific levels. However, this method also has limitations, due to the possible presence of several different copies of the ITS1 within a single bacterial chromosome, coupled with the difficulties in the interpretation of the results for ecological studies, and the incomplete databases for the ITS1 sequences.

Due to the higher differentiation power of the *rpoD* gene sequences, a primer set PsEG30F/PsEG790R was designed in our laboratory (Mulet et al. 2009). It was based on all of the *Pseudomonas rpoD* gene sequences available in databases that represented 35 species from all *Pseudomonas* intrageneric phylogenetic clusters. The primers PsEG30F/PsEG790R show only a few degenerations, precisely two for the forward and one for the reverse primer, thus increasing their specificity. The only non-*Pseudomonas* bacterial genus showing significant similarity to both primers when these sequences were checked against the databases belonged to the genus *Alcanivorax* but not to any other close phylogenetically related genus (Mulet et al. 2009).

The PsEG30F/PsEG790R primer set amplified the expected *rpoD* internal fragment of 760 bp of the 96 *Pseudomonas* type strains known at the time of the

experiments of Mulet and collaborators were done (2009). They also successfully amplified a well-characterized *Pseudomonas* collection consisting of more than 100 strains. The specificity of the primers was also verified by attempting the amplification of DNA from non-*Pseudomonas* strains. None of these control experiments resulted in the production of the amplicon (Mulet et al. 2009).

1.7.3 Molecular Typing

Usually in taxonomy, the strain differentiation within a species is considered a typing method. Most have been specifically designed for *P. aeruginosa* as epidemiological tools but may be useful also for other species. Several methods for serological, bacteriophage, pyocin and siderophore typing, among others, are available. In the present chapter, we will focus only on genome-based typing methods.

Genome fingerprints can be obtained by PCR amplification of genome regions by random designed primers (BOX, ERIC, REP, etc.), and the resulting amplicons are resolved by gel electrophoresis (Grundmann et al. 1995). In other methods, specific regions are amplified, and the amplicon is digested with several enzymes producing the corresponding patterns (e.g., ARDRA, amplified ribosomal DNA restriction analyses). Another approach consists in the digestion of total DNA of the strain, the separation of the oligonucleotides by conventional gel electrophoresis and the hybridization by Southern blot with specific DNA probes (e.g., ribotyping with RNA probes). DNA fragments obtained after digestion of total DNA with endonucleases can be also resolved by pulse-field gel electrophoresis (PFGE) generating strain-characteristic restriction patterns. PFGE is still considered by several authors to be the “gold standard” for typing *Pseudomonas* strains. However, other DNA sequence-based techniques are now available.

Multilocus sequence typing (MLST) is a relatively new typing technique that is becoming popular due to the ease of data analysis (Johnson et al. 2007). Although some studies showed that macrorestriction fragment analysis of whole DNA by pulse-field gel electrophoresis (PFGE) had a higher discriminatory ability than MLST, it has the advantage that it gives better information about the clonal relationships of isolates than PFGE (Johnson et al. 2007). The procedure proposed by Curran et al. (2004) for *P. aeruginosa* is based on the nucleotide sequence analysis of seven housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*). Criteria for the selection of these genes are their biological role, size of the amplicon (less than 600 bp), location in the chromosome, suitability for primer design, and sequence diversity. An arbitrary number is given to each distinct allele within a locus (gene). Therefore, each isolate receives seven numbers (allele profile) that represents a sequence type (ST). Each ST is numbered in order of appearance. More than 2200 STs are deposited in the “*Pseudomonas aeruginosa* MLST database” (<http://pubmlst.org/paeruginosa/>). MLST is rapidly becoming the gold standard for strain typing and it is the only method that permits precise phylogenetic analyses (Guttman et al. 2008).

A similar MLST procedure has been proposed for *P. fluorescens*. Around hundred STs have been already defined. The seven housekeeping genes analyzed are *glnS*, *gyrB*, *ileS*, *nuoD*, *recA*, *rpoB*, and *rpoD* as proposed by Andreani et al. (2014). The online database specific for *P. fluorescens* is available at: <http://pubmlst.org/pfluorescens/>.

1.8 Detection of *Pseudomonas* in Environmental DNA by *rpoD* Selective Primers

It has been well demonstrated that only a small fraction of the bacteria present in an environmental sample is recovered by culture-dependent techniques. Although *Pseudomonas* strains are easily isolated, several molecular methods have been proposed for the direct detection and identification of *Pseudomonas* in environmental samples. Besides the metagenomic studies, PCR-based strategies to evaluate the presence of *Pseudomonas* populations in environmental samples by culture-independent methods have been published recently.

As mentioned before, Widmer and collaborators designed a set of primers (Ps-for/Ps-rev) based on 16S rDNA. The objective of the design of the Ps-for and Ps-rev primers was to develop a PCR approach that would allow the selective detection of *Pseudomonas* (*sensu stricto*) in environmental samples (Widmer et al. 1998). However, the limited resolution power of the 16S rRNA does not allow deep species diversity studies. Another strategy was developed by Lloyd-Jones et al. (2005) for quantification of the *Pseudomonas* population in soil by a fluorogenic PCR assay. Specific forward and reverse primers were designed to amplify a 65-bp amplicon from *Pseudomonas* 16S rRNA genes and an MGB-TaqMan probe (Pp, 19 nucleotides long) to allow quantification of this amplicon by real-time PCR assay of soil DNA extracts. The authors detected *Pseudomonas* populations in the soils studied in the order of magnitude of 10^7 – 10^8 *Pseudomonas* cells per g dry weight soil, which represents 0.1–1 % of the total bacterial population. In contrast, the colony former units, detected in the same samples by a culture-dependent assay on *Pseudomonas* selective Gould S1 medium, ranged from less than 10^3 to 8×10^4 colonies per g dry weight soil. The authors concluded that “despite the large numbers of *Pseudomonas* that have been described, our knowledge of their diversity is constraint by an inability to cultivate the vast majority of this genus as it exists in the environment.”

The aforementioned set of *rpoD* primers PsEG30F/PsEG790R has been also used directly with environmental DNA to detect *Pseudomonas*. Total DNA of a fuel oil-contaminated sand sample was amplified with these primers. The amplicon was cloned and the *rpoD* insert was sequenced to identify the species present in the sample (Mulet et al. 2009, 2011). In fact, 46 of the 84 clones analyzed (55 %) belonged to the genus *Pseudomonas*. Clones related to the genus *Alcanivorax* were also detected. However, both of the *Pseudomonas* and *Alcanivorax* groups were clearly separated in different *rpoD* phylogenetic branches such that the clones could be easily differentiated and the sequences were ascribed to the corresponding species in the *rpoD* sequence. Although the primers were not genus specific, it

was concluded that the primers designed and tested were sufficiently selective for the detection of *Pseudomonas*.

Pyrosequencing of the *rpoD* amplicon was a further step in the sequencing effort. Total DNA extracted from a water sample of the Woluwe river (Belgium) and amplified with selective *rpoD* gene primers has been sequenced by pyrosequencing (Sánchez et al. 2014). Among a total of 14,540 reads, 6228 corresponded to *Pseudomonas rpoD* gene sequences by a BLAST analysis in the NCBI database. The selection criteria for the reads were sequences longer than 400 bp, an average *Q40* value greater than 25, and >85 % identity with a *Pseudomonas* species. Of the 6228 *Pseudomonas rpoD* sequences, 5345 sequences accomplished the established criteria for selection. Sequences were clustered by phylogenetic analysis and by the use of the QIIME software package. Representative sequences of each cluster were assigned by BLAST analysis to a known *Pseudomonas* species when the identity with the type strain was greater than 96 %. Twenty-six species distributed among 12 phylogenetic groups or subgroups previously described within the genus were detected by pyrosequencing. The predominant phylogenetic group within the *Pseudomonas* genus was the *P. fluorescens* group, as determined by culture-dependent and culture-independent analyses. In all analyses, a high number of putative novel phylospecies were found: ten were identified in the cultured strains and 246 were detected by pyrosequencing, indicating that the diversity of *Pseudomonas* species has not been fully described.

1.9 Future Prospects

Continuously, new bacterial species are described. As stated in the List of Prokaryotic Names with Standing in Nomenclature, in the period July 2013–June 2014, 928 new bacterial species/subspecies have been described and 63 % of them are from environmental origin. Six new *Pseudomonas* species have been described in 2013 and two more in 2014 at the moment of writing this chapter. All of them are from environmental origin. We can expect at least a similar increase in the next years. The development of novel culture-independent methods to study specifically *Pseudomonas* populations but also the metagenomic studies undertaken in many different habitats will help also in improving our understanding of the ecology of the genus. On the other hand, several genomes of *Pseudomonas* strains isolated from many different habitats are currently sequenced, and the comparative genomic analysis will decipher better the evolutionary history of the species under a phylogenomic perspective.

In the frame of the Genomic Encyclopedia of Bacteria and Archaea (GEBA) the Joint Genome Institute of the Department of Energy in the USA is sequencing thousands of bacterial and archaeal genomes from diverse branches of the tree of life (Wu et al. 2009). The GEBA type strain project is currently sequencing type strains with the aim to generate a comprehensive genomic encyclopedia of the validly named bacterial and archaeal species. The type strains serve as a fixed reference point for the assignment of bacterial and archaeal names and exhibit all

the relevant phenotypic and genotypic properties cited in the original published taxonomic circumscriptions. These sequences are unavoidable for developing phylogenomic taxonomy. Only recently, the whole-genome sequence of the type strain of *P. aeruginosa* has been published (Nakano et al. 2015). Once all *Pseudomonas* type strains have been sequenced, we will have a novel field for exciting taxonomic studies in the genus.

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Rachhpal S. Kahlon

Contents

2.1	Introduction	26
2.2	Structure of Outer Membrane	28
2.2.1	Phospholipids	28
2.2.2	Lipopolysaccharides (LPS)	29
2.2.3	Outer Membrane Proteins (Porins)	38
2.2.4	Outer Membrane Vesicles	51
2.3	Periplasm	54
2.4	Cell Wall	56
2.4.1	Molecular Structure	57
2.4.2	Cell Wall Biosynthesis	60
2.5	Inner Membrane (IM): Structure and Function	64
2.6	Biogenesis of Cell Envelope	66
2.7	Conclusion	70
	References	70

Abstract

Pseudomonas are typical gram-negative bacteria and have the unique envelope architecture comprising of two membranes, the outer membrane and inner (cytoplasmic) membrane, separated by thick viscous periplasmic space which houses thin layer of peptidoglycan, the cell wall. The multilayered cell envelope limits the cell size and protects from environmental stresses and performs important functions such as nutrient acquisition, adhesion, secretion, signaling, pathogenicity and efflux pumps for exclusion of antibiotics. The outer membrane has asymmetrical structure in which the inner leaflet is composed of phospholipid similar to the double-layered inner membrane which is universal. The outer leaflet is composed of lipopolysaccharide having three subunits, a glycolipid, lipid A

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which holds it in position, and a core polysaccharide which forms a link between the lipid A and the O-antigen which extends outwards. The outer membrane allows selective permeability through porins embedded in OM and is a host to several other proteins and enzymes. The peptidoglycan is thin and is held in position in the periplasm by lipoproteins which anchor it to the outer membrane, and some molecules extend all through the periplasm between IM and OM. Periplasm is the site for several biological activities such as polymerization of macromolecules and export of several surface proteins and other molecules. Precursors for these are synthesized in the cytoplasm or inner surface of the inner membrane and then transported to the periplasm for polymerization. The inner membrane is the typical phospholipid bilayer forming a mosaic of proteins for nutrient transport, energy generation, syntheses of precursors for cell wall, outer membrane, etc. Genomic and proteomic analyses show that envelope represents more than 1/3 of the ORFs and is host to a large number of enzymes and proteins involved in transport and enzymatic reactions and as structural proteins.

2.1 Introduction

Cell envelope is important from the point of view of both structure and physiology as the envelope limits the cell size, holds cellular contents and is responsible for the maintenance of cell shape. Besides this, it provides for cellular fluidity and is directly in contact with its surrounding environment for uptake and efflux reactions. In gram-negative bacteria, the cell envelope is a multilayered structure comprising of an outer membrane (OM) structure which itself is a bilayer, the peptidoglycan cell wall (CW) lying within the periplasmic space and the inner membrane (IM) composed of phospholipid bilayer embedded with several proteins and enzymes (Heppel 1967; Osborn et al. 1972). Microbial membranes are responsible for a plethora of processes such as the regulation of the movement of substances in and out of the cell, stabilizing protein structure for proper functioning of the membrane-bound enzymes and providing matrix for many biological reactions besides attachment and replication of chromosome. Ubiquitous bacteria like *Pseudomonas* are also capable of modulating their gene expression in response to wide range of environmental conditions for physiological and biochemical adaptations.

The uniqueness of the cell envelope in gram-negative bacteria is primarily attributed to lipopolysaccharide (LPS)-containing outer membrane. This layer delimits the zone outside the cytoplasmic membrane and regulates the passage of molecules into and out of the periplasmic space. The multilayered cell wall is made up of a variety of structural macromolecules which trap free molecules and ions. Interesting aspect of the cell wall is the continual synthesis for cell growth. The macromolecules for the cell envelope synthesis must be produced at the cytoplasmic membrane and suitably programmed to fit the appropriate layer (Salton 1994).

Freeze-etched studies of *P. aeruginosa* showed that the cell envelope is distinguishable into nine different layers, out of which five (L₁, L₃, L₅, L₇ and L₉) are

electron-dense and the rest are electron-transparent layers (Lickfield et al. 1972; Gilleland et al. 1973). The smoother layer, L₁, contains high lipid concentration; chemically it is mosaic of lipid bilayer comprising of phospholipid and lipopolysaccharide (L₁–L₃). LPS, antigenic structure projects outwards into the environment and is detected serologically in intact cells. In addition there are proteins such as lipoproteins and glycoproteins; some of these can be observed in electron-transparent layer, L₂. Beneath the outer membrane is the periplasmic space and contains some free protein moieties, while some others are covalently linked to outer membrane or to the mucopeptide component. Within the periplasmic region lies the mucopeptide which forms the cell wall. This may be associated with outer membrane and/or with cytoplasmic membrane. The next is the phospholipid bilayer, the cytoplasmic (inner) membrane. This is a mosaic of phospholipids and proteins. The cytoplasmic membrane bilayer is the homopolymer, i.e. two layers comprising of same types of phospholipids with hydrophilic polar ends facing cytoplasm and periplasm and the hydrophobic chains extending towards each other. This is in contrast to the asymmetrical structure of outer membrane in which the inner leaflet is composed of phospholipids similar to cytoplasmic membrane and the outer leaflet is primarily composed of complex glycolipids and lipopolysaccharide (LPS). The overall structure of the envelope is presented in Fig. 2.1.

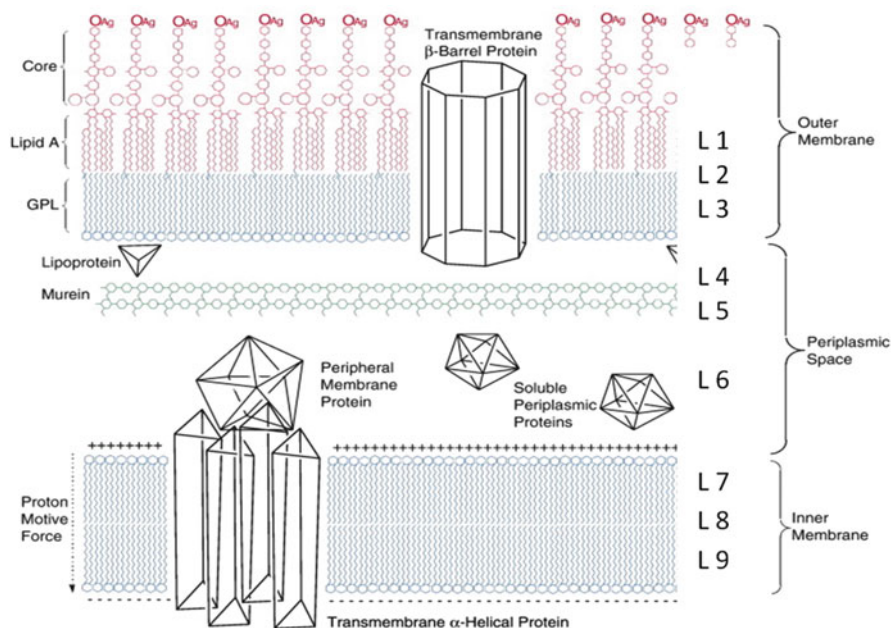


Fig. 2.1 Overall structure of the cell envelope of *Pseudomonas aeruginosa*, L1 to L9 correspond to the Lickfield et al. (1972) freeze-etched study, and L1 (LPS comprising of lipid A, core polysaccharide and O-antigen-O_{Ag}), L3 (GLP-glycerophospholipid), L5 (murein cell wall), L7 and L9 (IM-glycerophospholipid) are electron dense, and L2, L4, L6 and L8 correspond to electron-transparent layers, i.e. spaces between electron-dense layers (With permission Bishop, 2008)

2.2 Structure of Outer Membrane

Basically the outer membrane comprises of phospholipids, proteins and variable amount of lipopolysaccharide. Biophysical studies indicate that proteins and phospholipids form basic continuum, and the LPS is associated with inner and outer surfaces of the outer membrane (Cheng et al. 1971; Costerton et al. 1974; Silhavy et al. 2010). The double-layered outer membrane has asymmetrical structure, comprising of inner leaflet composed of phospholipids similar to those of the cytoplasmic membrane and the outer leaflet having more complex structure composed of glycolipid–lipopolysaccharide (LPS) complex and few phospholipids if any similar to the inner leaflet. Nearly 160 transmembrane proteins mostly having β -barrel structure are embedded in the outer membrane bilayer. Besides this there are lipoproteins which are usually associated with inner monolayer. The outer membrane proteins of *Pseudomonas* have been designated as “OPR” (outer membrane proteins) to differentiate these from OMP, the outer membrane proteins of *E. coli* (Bell and Hancock 1989; Hancock et al. 1990; Hancock 1991; Bitter 2003). These components impart specific properties such as selective permeability barrier, non-specific efflux, export of molecules and proteins and interaction with immune systems and environment. Besides resistance to antibiotics, OM is host to a number of enzymes and help in anchoring of appendages such as fimbriae and flagella (Hancock and Brinkman 2002; Blander et al. 2004).

2.2.1 Phospholipids

Phospholipids in the inner leaflet of the outer membrane are similar to cytoplasmic membrane of gram-negative bacteria. These are primarily phosphatidylethanolamine with small amounts of diphosphatidylglycerol (cardiolipin) and other acidic phospholipids. Under conditions of phosphate limitation, *P. fluorescens* also have been reported to contain ornithine amine lipids.

The fatty acid tails of phospholipids of *P. aeruginosa* are palmitic acid (16:0) as the major component and oleic acid (18:1) or 19-carbon cyclopropane as the next most important component. Predominant unsaturated fatty acid in pseudomonads is oleic acid, C18:1 instead of palmitoleic acid, C16:1. Higher concentration of longer fatty acids in outer membrane in pseudomonads is considered to be responsible for more rigid envelope as compared to other gram-negative bacteria (Nikaido and Hancock 1986). In addition to some other saturated fatty acids in the range from C10:0 to C17:0 which may be branched at the ends and unsaturated fatty acids in the range of 15 to 18, carbon atoms generally monounsaturated with *cis*-double bonds are produced by members of genus *Pseudomonas*. Some species also produce cyclopropane fatty acids (C17:▲ or C19:▲).

Depending upon the environmental stresses such as temperature, pH, heavy metal ions, organic solvents or nutrient limitation, bacteria can change their lipid profile. This can be achieved by modifying the *cis*-unsaturated fatty acids into their *trans*-isomers by *cis-trans* isomerase. Alternatively, they may undertake

differential synthesis of saturated fatty acids so as to increase their concentration in the membrane. Both the processes result in longer fatty acid tails and their tight packing enhances rigidity of cell membrane. Substitution of saturated fatty acids by unsaturated fatty acids results in increased permeability of outer membrane.

2.2.2 Lipopolysaccharides (LPS)

Lipopolysaccharide (LPS) is the major component of the outer leaflet of gram-negative bacteria and is a carbohydrate-lipid complex comprising of three domains, lipid A, which anchors LPS in the outer membrane, a short “core oligosaccharide” and variable “O-antigen”, a long-chain polysaccharide (Fig. 2.2). LPS being a component of the outer leaflet of the outer membrane plays an important role in the response of the organism to the environment with which it is in direct contact. Although there is overall similarity of LPS between *E. coli* and *P. aeruginosa*, striking differences exist in organization and chemical structure. As in enteric bacteria, pseudomonads have a common basic LPS molecule which may undergo modification in different strains as differences in the LPS structure have been

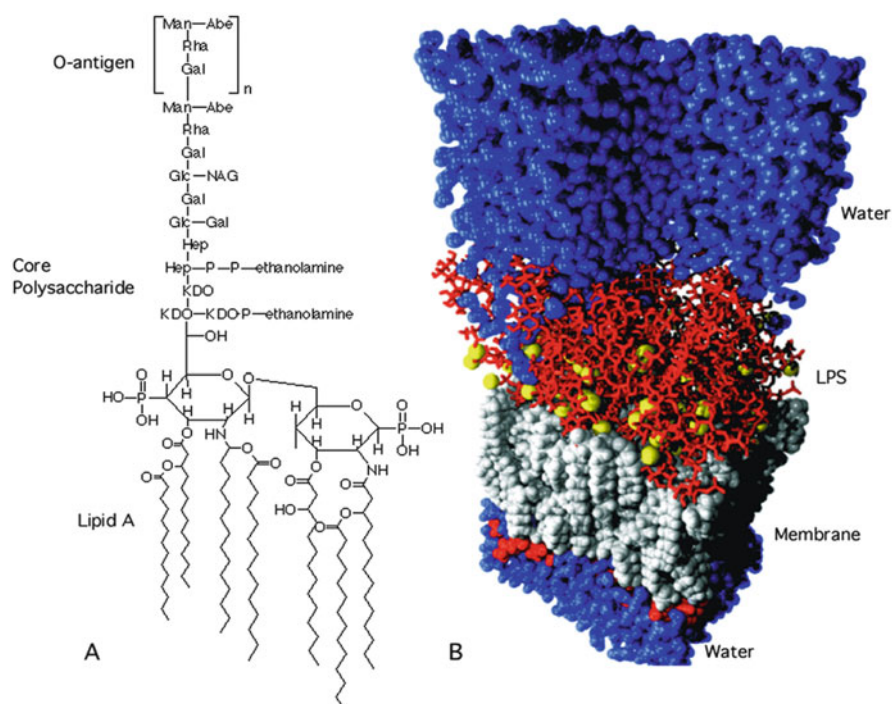


Fig. 2.2 (a) Chemical structure of lipopolysaccharide, comprising of three subunits, the lipid A, core polysaccharide and O-antigen; (b) the molecular model of LPS of *Pseudomonas aeruginosa* (Printed with permission from Paustian, 2013)

reported at the strain and species level. Amongst pseudomonads, the LPS (endotoxin) of *P. aeruginosa* has been extensively studied and plays an important role in virulence, and the O-polysaccharide chain is the target moiety for antibodies. Major emphasis has been on chemical structure and immunological properties. Antigenic typing of O-antigen showed 20 different serotypes of *P. aeruginosa* (Liu and Wang 1990; Burrows and Lam 1999). In *P. aeruginosa* two variables of LPS have been recognized, first a typical O-antigenic LPS also called “B-band LPS” (renamed O-specific antigen—OSA) and a minor LPS referred to as “A-band LPS” (renamed common polysaccharide antigen—CPA—or common O-polysaccharide chains). CPA is homopolymer of D-rhamnose (D-Rha) and elicits weak antigenic response and is usually 70 sugar residue long chains (Burrows et al. 1996). The endotoxic lipid A moiety which forms a major component of the outer surface monolayer is inserted into the outer monolayer of the outer membrane. In pseudomonads the lipid A is composed of di-phosphorylated di-glucosamine and has a highly conserved structure amongst *Pseudomonas* sp. Lipid A is inserted into the membrane via several attached fatty acids. In *P. aeruginosa* the lipid A is derived from a β -1,6-linked disaccharide of glucosamine. The hydroxyl groups of glucosamine are esterified with long-chain fatty acids, while 3OH 12:0 is joined by amide linkage to the amino group of glucosamine. The hydroxyl group of this hydroxyl acid is substituted with 16:0 and 2OH 12:0 fatty acid. Within the species this region is similar in composition and is composed of various sugars and phosphate molecules that along with anionic sugars and lipid A phosphates serve as divalent cation binding sites for LPS. Part of the O-polysaccharide comprising of tri- and tetra-polysaccharide is exposed to external environment and is responsible for immunogenic properties (Raetz and Whitfield 2002; Bystrova et al. 2003; Knirel et al. 2006). Thus *P. aeruginosa* LPS is similar to enteric bacterial LPS comprising of three domains, the biphosphorylated D-glucosamine disaccharide–lipid A complex; a core comprising of nine- or ten-sugar, branched oligosaccharide; and serologically diverse O-antigenic chain. The core is covalently attached to lipid A complex on one end and the O-antigen on the other. However, differences lie in the fact that *P. aeruginosa* LPS contains large number of phosphate residues, amino acid L-alanine in core and some unusual sugars and amino compounds in O-chain which are not present in enteric bacteria. LPS heterogeneity can be achieved through variations in the sugar moieties within the O-antigen repeating unit, the type of the glycosyl linkages, the addition of noncarbohydrate moieties to the O-antigen (i.e. O-acetylation) and the ratio of smooth versus rough LPS molecules. Most strains of *P. aeruginosa* have a capping frequency (core-lipid A molecules substituted with long-chain O-antigen) of between 0.2 and 14 %. Growth at elevated temperatures decreases O-specific antigen chain length as the temperature increases.

2.2.2.1 Lipid A

Lipid A is the inner portion of LPS. This moiety consists of an N- and O-acylated di-glucosamine biphosphate backbone $[4\text{-P-}\beta\text{-GlcP}^{\text{II}}(1\rightarrow6)\alpha\text{-X-glcP}^{\text{I}}(1\text{-P})]$. The general acylation patterns are conserved within different strains and species, but variability is observed in respect of the number of primary acyl groups and

number, nature and position of secondary acyl groups (Fig. 2.2). The disaccharide is anchored into the outer membrane by six or seven fatty acyl chains linked through either ester or amide linkages (Nikaido and Hancock 1986). Predominantly these are hydroxyl fatty acids and only small amount of saturated fatty acids may be present. Important hydroxy fatty acids are 3OH dodecanoate ($C_3OH-12:0$) and 2OH dodecanoate ($C_2OH-12:0$) and traces of 3OH decanoate ($C_3OH-10:0$), while Enterobacteriaceae primarily contain 3OH tetradecanoate ($C_3OH-14:0$). Shorter fatty acid chains in *Pseudomonas* may be responsible for higher fluidity of its outer membrane as compared to other gram-negative bacteria. Lipid A structure varies with growth conditions; in laboratory strains of *P. aeruginosa*, mostly five (~75 %) and six (~25 %) fatty acid substituents were identified on the glucosamine backbone (Kulshin et al. 1991). The hydroxyl groups of these fatty acids may be substituted by a palmitate ($C_{16:0}$) or 2OH dodecanoate ($C_2OH-12:0$).

Therefore, *Pseudomonas* LPS may contain fatty acyl chains of 10–18 carbon atoms which may be saturated or monounsaturated. *Pseudomonas rubescens* has been reported to contain terminally branched fatty acids. Variations in fatty acyl chains also serve as criteria for identification of *Pseudomonas*. Lipid A may be further modified by addition of a cationic 4-amino-4-deoxy-L-arabinose (ara4N) sugar residue in non-stoichiometric quantity at the 1'- or 4'-phosphate group (Ernst et al. 2003; Trent 2004).

The biological role of lipid lies in its ability to induce innate immune system by interaction with Tol-like receptor 4 (TLR 4) on the surface of immune cells. O-palmitoylation modulates signal via TLR-4 and is involved in the adaptation of *P. aeruginosa* to chronic infection of the human airways. Addition of polar Ara4N to phosphate groups induces resistance to cationic antimicrobial peptides in response to environmental conditions. Glycosylation with Ara4N and O-palmitoylation may play an important role in persistence of *P. aeruginosa* in cystic fibrosis-associated lung infection. Shorter OH fatty acid tails of *Pseudomonas* lipid A are responsible for moderately decreased toxicity of lipid A as compared to lipid A enteric bacteria.

The biosynthesis of lipid A has been studied in detail in *E. coli* and this pathway is assumed to be generally conserved in *P. aeruginosa*. This assumption is based largely on the identification of homologues of the *E. coli* genes in the *P. aeruginosa* genome (King et al. 2009), but the majority of lipid A biosynthetic steps have not been directly investigated. The subject has been recently reviewed by Raetz and Whitfield (2002) and Trent (2004).

Environmental conditions influence structure of lipid A resulting in greater resistance to antimicrobial peptides. Two-component PhoP–PhoQ regulatory system acts in response to divalent cation (Ca^{++} , Mg^{++}) concentration to promote in vivo survival of *P. aeruginosa* in a manner similar to *Salmonella enterica* serovar Typhimurium (Macfarlane et al. 1999; McPhee et al. 2006, 2009).

2.2.2.2 Core Oligosaccharide

The core oligosaccharide comprising of sugar molecules—galactosamine, rhamnose, glucose, L-glycero-D-mannoheptose and the unique octose, 2-keto-3-deoxy-

octulosonic acid (KDO)—is attached to lipid A. The KDO residues lie at the inner end and is extended to a few heptose residues some of which are phosphorylated or carry phosphoethanolamine or pyrophosphoethanolamine substituents. The outer part mostly consists of hexoses; thus, the core oligosaccharide may be divided into outer core and inner core. The inner core in *P. aeruginosa* is usually composed of heptose, two molecules of L-glycero-D-manno-heptose (Hep^I and Hep^{II}) and two KDO residues (Kdo^I and Kdo^{II}). The core may be substituted with alanine and phosphate molecules. *Pseudomonas* core region contains about two times more phosphate than found in Enterobacteriaceae. The phosphate molecules in the core region contribute to the barrier function of the outer membrane. Three phosphorylation sites are positions 2 and 4 on Hep^I and position 6 on Hep^{II}. Phosphorylation of LPS results in negative charge on the cell surface which is partially neutralized by Mg⁺⁺ ions.

Phosphorylation of LPS also plays a role in variability and intrinsic resistance to antibiotics (Walsh et al. 2000). Besides, this phosphorylation of the inner core contributes to the stabilization of the outer membrane by formation of intermolecular ionic bridges involving Ca⁺⁺ and Mg⁺⁺. Each phosphorylation site may be occupied by mono-, di- or even triphosphate. Ethanolamine in the core oligosaccharide of *P. aeruginosa* may play a role in resistance to cationic antimicrobial peptides.

Two structurally similar outer core glycoforms, 1 and 2, have been reported in smooth laboratory strains and clinical isolates of *P. aeruginosa* in comparable amounts. Both glycoforms share the same outer core tetrasaccharide consisting of one D-galactosamine (GalN) and three D-glucose (Glc^I–Glc^{III}) residues but differ in position of L-rhamnose (Rha) residues attached to D-glucose (Knirel et al. 2006).

GalN is substituted on position 2 by an alanyl (ala) group or some truncated core with acetyl group. O-acetylation of the outer core sugars is common in *P. aeruginosa* particularly isolated from cystic fibrosis patients. O-acetylation of bacterial surface glycopolymers affects their binding and gel-forming ability besides hydrophobicity of cell surface and thus plays a role in resistance to phagocytic killing. The outer core OS of *P. aeruginosa* exists in two structurally distinct glycoforms, viz. capped or uncapped (King et al. 2009). They differ in position and linkage of a Rha residue in each structure. The capped glycoform is covalently attached to O-antigen on Rha^B that 1,3 linked to Glc^I, whereas the uncapped cannot be substituted with O-antigen, and it contains a L-Rha^A that is 1,6 linked to Glc^{II} (Fig. 2.3).

- (1) Genetic analysis of *Pseudomonas aeruginosa* shows a cluster of genes, *pa4996–pa5012*, coding proteins involved in core OS biosynthesis (Table 2.1). Apart from the presence or absence of the Glc^{IV} residue as a terminal sugar in uncapped core OS, the structures of core OS amongst *P. aeruginosa* strains are relatively conserved with no strain-to-strain variability in sugar composition. This observation is consistent with the discovery that the genes in the locus for core biosynthesis are well conserved amongst *P. aeruginosa* strains. The gene products in this locus show relatively high amino acid identity, ranging from

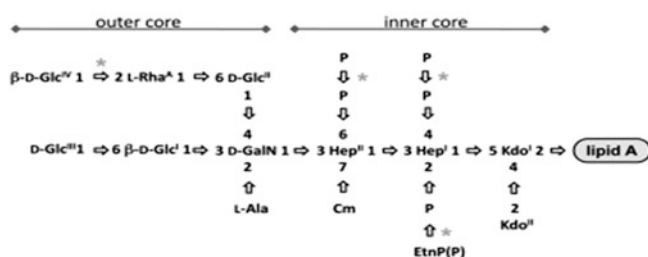
A Uncapped core**B Capped core**

Fig. 2.3 The structures of the uncapped core oligosaccharide (a) and capped core oligosaccharide (b). *Ala* alanine, *Cm* carbamoyl, *Etn* ethanolamine, *GalN* 2-amino-2-deoxy-galactose (galactosamine), *Glc* glucose, *Hep* 1-glycero-D-manno-heptose, *Kdo* 3-deoxy-D-manno-oct-2-ulosonic acid, *Rha* rhamnose. Asterisks show variable substitution sites (Lam et al. 2011)

77 to 100 %. In addition to genes that encode kinases and heptosyltransferases, this cluster contains genes that are involved in the transportation of lipid A and ligation of O-Antigen to the core (*msbA* and *waaL*, respectively). Two other genes, *pa5002* and *pa5003*, have not been characterized. Two transferase genes, *migA* and *waaA*, are important for core OS biosynthesis but are localized outside the usual core LPS gene locus. The *waaA* gene encodes a putative Kdo transferase. As not all of the *P. aeruginosa* strains possess Glc^{IV} as a terminal sugar residue in their uncapped core OS, not all of the *P. aeruginosa* strains have the *wapB* gene. For instance, this gene is absent from the sequenced genome of *P. aeruginosa* PA7. The subject has been reviewed in detail (King et al. 2009; Lam et al. 2011).

Another variability in the structure of the *P. aeruginosa* core OS arises from the different degrees of phosphorylation (including ethanolamine phosphate) and acetylation patterns. However, the variability of these phosphatidyl or acetyl substitutions is non-stoichiometric, and the genetic elements that account for these minor substitutions are not known. The regions of the core oligosaccharides (OS) that are conserved include the carbohydrate backbone of the inner region, the two glycoforms forming outer regions, the three phosphorylation sites of Hep^I at positions 2 and 4 and Hep^{II} at position 6, 7-O-carbamoylation of Hep^{II} except one strain and N-acetylation of GalN with L-alanine.

Table 2.1 Genes and enzymes of the core oligosaccharide (OS) biosynthesis in *P. aeruginosa* (Lam et al. 2011)

Gene	Proposed/demonstrated function	References
Core biosynthesis gene cluster (<i>pa4996-pa5012</i>)		
<i>hldE/</i> <i>pa4996</i>	Heptose biosynthesis	King et al. (2009)
<i>msbA/</i> <i>pa4997</i>	Transport lipid A-core	Ghanei et al. (2007)
<i>pa4998</i>	Kinase	King et al. (2009)
<i>waaL/</i> <i>pa4999</i>	O-antigen ligase	Abeyrathne and Lam (2007)
<i>wapR/</i> <i>pa5000</i>	Glycosyltransferase (Rha ^B)	Poon et al. (2008)
<i>pa5001</i>	Glycosyltransferase	King et al. (2009)
<i>pa5002</i>	Unknown	
<i>pa5003</i>	Unknown	
<i>wapH/</i> <i>pa5004</i>	Glycosyltransferase (Glc ^{II})	Matewish (2004)
<i>wapO/</i> <i>pa5005</i>	Carbamoyltransferase	(King et al. (2009)
<i>pa5006</i>	Kinase	King et al. (2009)
<i>wapQ/</i> <i>pa5007</i>	Heptose kinase	Walsh et al. (2000)
<i>wapP/</i> <i>pa5008</i>	Heptose kinase	Walsh et al. (2000), To (2006)
<i>waaP/</i> <i>pa5009</i>	Heptose kinase: position 4 of HepI	Walsh et al. (2000), Zhao and Lam (2002), Zhao et al. (2002)
<i>wapG/</i> <i>pa5010</i>	Glycosyltransferase (GalN)	
<i>waaC/</i> <i>pa5011</i>	Glycosyltransferase (Hep ^I)	De Kievit and Lam (1997)
<i>waaF/</i> <i>pa5012</i>	Glycosyltransferase (Hep ^{II})	De Kievit and Lam (1997)
Genes located outside of the core biosynthesis cluster		
<i>wapB/</i> <i>pa1014</i>	Glycosyltransferase (Glc ^{IV})	Kocíncová et al. (2011)
<i>migA/</i> <i>pa0705</i>	Glycosyltransferase (Rha ^A)	Poon et al. (2008)
<i>waaA/</i> <i>pa4988</i>	Glycosyltransferase: (KdoI, KdoII)	King et al. (2009)

Proteins MsbA and WaaL are involved in processes other than core biosynthesis

Besides these conserved features, the following optional core features have been reported:

1. Glycosylation of Rha with Glc^{IV} in glycoform 1
2. Phosphorylation of Hep^{II} at position 4 (a minor phosphorylation site)

3. Attachment of ethanolamine phosphate (EtnP_i) to phosphate group at position 2 of Hep^I giving rise to ethanolamine pyrophosphate (EtnPP)
4. The presence of diphosphate (triphosphate) groups at each phosphorylation sites
5. O-acetylation of the outer core region

The non-obligatory substituents of the core are present in non-stoichiometric amounts, which vary from strain to strain.

2.2.2.3 O-Polysaccharide (OPS)

The long-chain O-polysaccharide (OPS), earlier called O-antigen of *P. aeruginosa*, is antigenic in nature and elicits antibody formation and 20 different serotypes have been identified. This is the most diverse region of the LPS and varies with respect to sugar composition, linkage, sequence and branch lengths. A typical *Pseudomonas* O-polysaccharide consists of repeating units of 3–5 sugars such as glucosamine, glucose, rhamnose, fucosamine and often aminohexuronic acids such as D-quinovosamine, 2-imidazolinomannuronic acid and 2,3-diacetamido-2,3-dideoxyhexuronic acid (Meadow 1975; Yokota et al. 1987). *Pseudomonas aeruginosa* possess two types of O-polysaccharide (OPS) referred to as common polysaccharide antigen CPA (earlier called A-band LPS) and O-specific antigen OSA (previously B-band LPS). CPA is common for both R-type and S-type strains of *P. aeruginosa* and is produced by majority of strains of *P. aeruginosa* and some other pseudomonads. This is a homopolymer of D-rhamnose arranged as trisaccharide repeating unit, $[\rightarrow 3)\text{-}\alpha\text{-D-Rha-(1}\rightarrow 2)\text{-}\alpha\text{-D-Rha-(1}\rightarrow 3)\text{-}\alpha\text{-D-Rha-(1}\rightarrow]_n$, with minor amounts of other sugars as well as rhamnan repeating unit and probable lipid A-core sugars; the following have been identified: 3-O-methyl-6-deoxyhexose and xylose; 3-O-methylrhamnose, ribose, mannose and 3-O-methylhexose or mannose, GlcNAc and small amounts of O- and N-acetylation (Yokota et al. 1987; Hao et al. 2013). Monoclonal antibodies reactive against polyrhamnose fraction react with many different serotypes indicating that there is common core antigen. CPA fraction of OPS elicits weak antibody response and is low in phosphate and amino sugars and does not react with serotype-specific monoclonal antibodies (Rivera and McGroarty 1989; Knirel et al. 2001) but contains sulphate instead of phosphate. The CPA chains are usually approximately 70-sugar unit long.

A cluster of eight genes (*rmd* to *wbpZ*, *pa5447*–*pa5454*) in *P. aeruginosa* PAO1 has been identified for the biosynthesis of CPA. Three of the proteins encoded by these genes, WbpW, Gmd and Rmd, are involved in the biosynthesis of the precursor nucleotide sugar, GDP-D-Rha. Three putative glycosyltransferases, WbpX, WbpY and WbpZ, have been proposed to be involved in the assembly of the trisaccharide D-Rha repeating unit. Wzm and Wzt form the ABC transporter for the translocation of undecaprenol-linked CPA polysaccharide (PS) to the periplasmic side of the cytoplasmic membrane (Rocchetta et al. 1998a, b). The involvement of *wzm* and *wzt* imparts the biosynthesis of CPA into the particular category of ABC transporter-dependent pathways. Based on bioinformatic evidence, another five-gene cluster (*pa5455*–*pa5459*) lying next to cluster *pa5447*–*pa5454* is involved in CPA biosynthesis and modification (Hao et al. 2013). Thus, the genes are clustered

Table 2.2 Enzymes involved in synthesis of common polysaccharide antigen (Hao et al. 2013)

Gene	Related proteins	Key reference
<i>pa5447</i>	Glycosyltransferase (GT-4)	Rocchetta et al. (1998a, b)
<i>pa5448</i>	Glycosyltransferase (GT-4)	Rocchetta et al. (1998a, b)
<i>pa5449</i>	Glycosyltransferase (GT-4)	Rocchetta et al. (1998a, b)
<i>pa5450</i>	ABC transporter	Rocchetta and Lam (1997)
<i>pa5451</i>	ABC transporter	Rocchetta and Lam (1997)
<i>pa5452</i>	D-Man-6-phosphate isomerase/GDP-D-Man pyrophosphorylase	Rocchetta et al. (1998a, b)
<i>pa5453</i>	GDP-D-Man 4,6-dehydratase	King et al. (2009)
<i>pa5454</i>	GDP-D-Rha synthase	King et al. (2009)
<i>pa5455</i>	Glycosyltransferase (GT-4)	Hao et al. (2013)
<i>pa5456</i>	Glycosyltransferase (GT-4)	Hao et al. (2013)
<i>pa5457</i>	Methyltransferase	Hao et al. (2013)
<i>pa5458</i>	Acetyltransferase	Hao et al. (2013)
<i>pa5459</i>	Methyltransferase	Hao et al. (2013)
<i>algC/ pa5322</i>	Phosphomannomutase/phosphoglucomutase	Zierlinski et al. (1991)

in two adjacent operons. Gene sequence analysis indicated that *pa5455* and *pa5456* encode putative glycosyltransferases. The *pa5458* gene encodes a protein with a conserved acetyltransferase domain. *PA5457* and *PA5459* contain conserved methyltransferase domains and show sequence similarity to *E. coli* O8 O-antigen terminator protein WbdD. Presumably the *pa5455*–*pa5459* gene cluster is part of the CPA biosynthesis locus encoding functions for biosynthesis and chain length regulation of the polymer (Table 2.2).

The other form of O-antigen named O-specific antigen (OSA) is a heteropolymer with three to five distinct sugars in repeat units. The structures of the biological repeating units have been elucidated for most of the *P. aeruginosa* IATS serotype O-specific antigens, and each has a 2-acetamido-2-deoxy-D-fucose (D-FucNAc), a 2-acetamido-2-deoxy-D-quinovose (D-QuiNAc) or a derivative of these at the reducing terminus (Bystrova et al. 2006). The presence of these different sugars at the reducing termini raised the question of the specificity of WbpL enzymes from different serotypes for the sugar substrate. OSA is the major antigen and highly immunogenic and responsible for different serotypes of *P. aeruginosa*; presently 20 different serotypes have been identified. The complexity and diversity of OSA produced by *P. aeruginosa* is based on the heterogeneity of the chain length of O-Ag present on the cell surface used to “cap” the core oligosaccharide. The majority of the core oligosaccharide molecules on the cell surface are not capped and are referred to as rough. Only about 10 % of molecules on the cell are capped

and hence called O-polysaccharide (OS). These smooth molecules are of variable length and show a “ladder banding” pattern when LPS from *P. aeruginosa* is analysed by silver-stained SDS-PAGE gel (Rocchetta et al. 1998a, b; Rocchetta et al. 1999; Poon et al. 2008).

Rough mutants of *P. aeruginosa* are avirulent and do not cause disease in experimental animals, indicating that O-polysaccharide is required for pathogenicity. Chain length may vary from single repeat unit to considerably longer chains than the CPA side chain in different serotypes.

Biosynthesis of O-antigen has been elucidated by Whitfield (1995) and CPA and OSA are assembled by different mechanisms. This has been substantiated by De Kievit and Lam (1997) and Rocchetta and Lam (1997). The CPA synthesis follows the “ABC transporter-dependant pathway” and OSA follows “Wzy-dependent pathway”.

According to both models, the O-polysaccharide sugars are assembled on an isoprenyl lipid carrier by cytoplasmic glycotransferases, and the completed O-polysaccharide chains are ligated to lipid A core in periplasm. However, two mechanisms are distinct with respect to steps involved. In ABC transporter-dependent pathway for CPA biosynthesis, the O-chain is fully assembled on the cytoplasmic side of the inner membrane (IM) and then exported to the periplasm. On the contrary for OSA biosynthesis by Wzy-dependent pathway, the O-repeating units are individually flipped across the inner membrane and polymerized in the periplasm by *wzy* gene product (Table 2.3).

Initiation of O-polysaccharide synthesis in both pathways takes place by transfer of a sugar-1-phosphate to undecaprenyl phosphate. This generates an undecaprenyl-pyrophosphoryl-linked glycan which can be extended by the action of glycosyl transferases. The initiating sugar-1-phosphate transferase in *P. aeruginosa* is encoded by *wbpL* located in OSA gene cluster and is required for synthesis of both CPA and OSA (Rocchetta et al. 1998a, b). Repeating units of O-specific antigen having a 2-acetamide-2-deoxy-D-fucose (D-FucNAC), a 2-acetamideo-2-deoxy-D-quinovose (D-QuiNAC) or a derivative of these at different reducing ends is incorporated. Gene *wbpM* coding for nucleotide sugar epimerase/dehydratase is conserved in all serotypes of *P. aeruginosa* and located at the distal end of OSA biosynthetic locus. This is involved in the synthesis of deoxy sugars (D-FucNAC) and D-QuiNAC and their derivatives. This gene is not required for CPA biosynthesis.

Subject of biosynthesis of O-polysaccharide has been reviewed by King et al. (2009, 2010), Ivanov et al. (2011), Islam et al. (2011) and Hao et al. (2013), and gene clusters have been elucidated (Lam et al. 2011).

The assembled O-unit is not only expressed in the LPS but also via glycosylation of pilin, the major glycoprotein of polymeric pili of *P. aeruginosa*. The genes involved in the synthesis of O-antigen are clustered in the *wbp* region of chromosome (Fig 2.4) (Lam et al. 2011; Hao et al. 2013).

Table 2.3 *Pseudomonas aeruginosa* PAO1 serotype 05 O-specific antigen (OSA) biosynthesis cluster (Lam et al. 2011)

Gene	Function	Reference
<i>wzz/</i> <i>pa3160</i>	OSA chain length regulator	Burrows et al. (1997), Daniels et al. (2002)
<i>wbp/</i> <i>pa3159</i>	UDP- <i>N</i> -acetyl-D-glucosamine 6-dehydrogenase	Miller et al. (2004)
<i>wbp/</i> <i>pa3158</i>	UDP-2-acetamido-2-deoxy-D-glucuronic acid 3-dehydrogenase	Westman et al. (2009)
<i>wbpC/</i> <i>pa3157</i>	Possible O-acetyltransferase	
<i>wbpD/</i> <i>pa3156</i>	UDP-2-acetamido-3-amino-2,3-dideoxy-D-glucuronic acid <i>N</i> -acetyltransferase	Wenzel et al. (2005), Westman et al. (2009)
<i>wbpE/</i> <i>pa3155</i>	UDP-2-acetamido-2-dideoxy-D-ribo-hex-3-uluronic acid transaminase	Westman et al. (2009)
<i>wzy/</i> <i>pa3154</i>	OSA α -polymerase	De Kievit et al. (1995), Islam et al. (2010), Islam et al. (2011)
<i>wzx/</i> <i>pa3153</i>	OSA unit flippase	Burrows and Lam (1999), Islam et al. (2010)
<i>hisH2/</i> <i>pa3152</i>	Imidazole glycerol phosphate synthase subunit	King et al. (2009)
<i>hisF2/</i> <i>pa3151</i>	Imidazole glycerol phosphate synthase subunit	King et al. (2009)
<i>wbpG/</i> <i>pa3150</i>	Amidotransferase	
<i>wbpH/</i> <i>pa3149</i>	Glycosyltransferase	
<i>wbpI/</i> <i>pa3148</i>	UDP- <i>N</i> -acetylglucosamine 2-epimerase	Westman et al. (2009)
<i>wbpJ/</i> <i>pa3147</i>	Glycosyltransferase (GT-4)	
<i>wbpK/</i> <i>pa3146</i>	NAD-dependent epimerase/dehydratase	
<i>wbpL/</i> <i>pa3145</i>	Glycosyltransferase (initiating glycosyl-1-P transferase)	Rocchetta et al. (1998a)
<i>wbpM/</i> <i>pa3141</i>	Nucleotide sugar epimerase/dehydratase	Creuzenet and Lam (2001)

2.2.3 Outer Membrane Proteins (Porins)

Outer membrane plays an important role in physiology of bacterial cell by acting as a selective permeability barrier, excluding potentially harmful molecule secretion of molecules, and as specific receptor complexes. Besides the pili, flagella, LPS and capsules are also anchored to the cell via outer membrane. It is both way true that molecules taken from the environment have to cross the outer membrane as well as other components of the cell envelope to reach the cytoplasm, so is the case for secretory molecules to reach the exterior of the cell. These functions are carried out

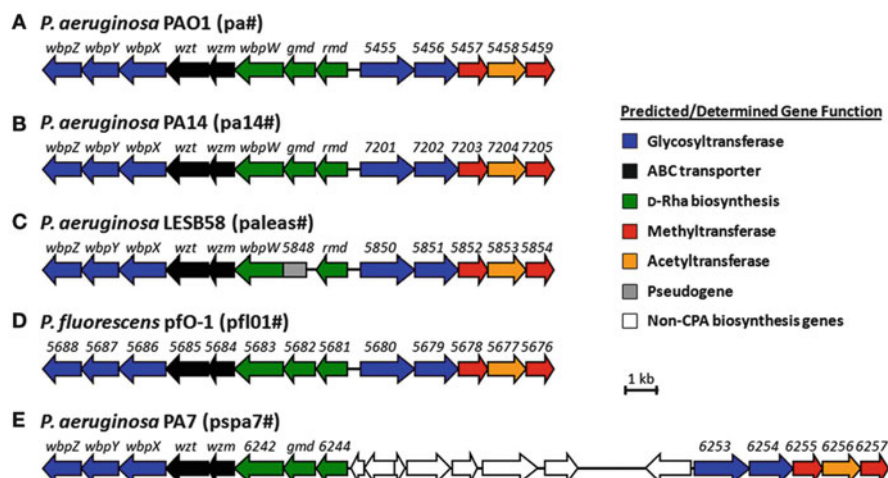


Fig. 2.4 Comparative gene clusters of CPA biosynthesis of different strains of *Pseudomonas* (Lam et al. 2011)

through specific and non-specific pores, specific receptor complexes and hydrophobic pathways. The lipoproteins Opr I, Opr L and Tol are involved in the structure and maintenance of cell shape. Porins are responsible for the selective uptake of nutrient substrates and other molecules. The efflux and secretion porins help in the removal of toxic compounds, proteins, etc. Some proteins located in the outer membrane also act as adhesion antigens and receptors for bacteriocins and bacteriophages. Comprehensive list of outer membrane proteins has been provided by Hancock and Brinkman (2002), Schulz (2002), www.pseudomonas.com and www.cruir.ubc.ca/bobh/omps/ (Table 2.4).

Pseudomonas outer membranes show low permeability equivalent to about 8 % of that of *E. coli* but show large exclusive limit, i.e. permitting passage even up to the molecules measuring 3000 Da compared to 500 Da for *E. coli* (Nikaido and Hancock 1986; Nikaido 2003). The porin channels mediate selective uptake of compounds ranging from small nutrient molecules to large iron-siderophore complexes. A number of efflux and secretion systems are involved in the export of toxic compounds, proteins, DNA, virulence factor, etc. Outer membranes of *P. aeruginosa* act as a strong barrier of antibiotics and other large hydrophobic molecules. Hydrophilic molecules are taken up through water-filled channels of porins. The porin channels of *P. aeruginosa* are considered narrow or highly specialized to allow passage of few substrates larger than monosaccharide (200 Da).

Low permeability has also been associated with antibiotic resistance, but alone is not enough, and enzymes such as β -lactamase located in the periplasmic space hydrolyse the β -lactam antibiotics that are transported at the low rate.

Table 2.4 List of major porins and related proteins of outer membrane of *Pseudomonas aeruginosa* (Hancock and Brinkman 2002; www.pseudomonas.com)

General porins			
<i>oprF</i>	PA1777	OprF	Major porin/Structural porin
<i>oprG</i>	PA4067	OprG	OprG of <i>P. putida</i> 70 % similarity with OprG of <i>P. aeruginosa</i>
<i>oprB</i>	PA3186	OprB	Glucose/carbohydrate porin, protein D1
<i>oprD</i>	PA0958	OprD	Basic amino acid/peptide/imipenem porin protein D2
<i>algE</i>	PA3544	AlgE	Alginate synthesis
Specific porins			
<i>fliF</i>	PA1101		Flagella M ring protein
<i>oprB</i>	PA3186	OprB	Glucose/carbohydrate porin, protein D1
<i>oprC</i>	PA3790	OprC	Copper transport porin
<i>oprD</i>	PA0958	OprD	Basic amino acid/peptide/imipenem porin protein D2
<i>oprE</i>	PA0291	OprE	Anaerobically induced porins, E1 & F
<i>oprF</i>	PA1777	OprF	Major porin/structural porin
<i>oprG</i>	PA4067	OprG	Outer membrane protein
<i>oprH</i>	PA1178	PhoP/Q	Low Mg ⁺⁺ inducible OM protein H1
<i>oprI</i>	PA2853	OprI	Outer membrane lipoprotein
<i>oprL</i>	PA0973	OprL	Peptidoglycan-associated lipoprotein
<i>oprO</i>	PA3280	OprO	Pyrophosphate-specific porin
<i>oprP</i>	PA3279	OprP	Pyrophosphate-specific porin, protein D
<i>oprA</i>	PA2688	PfeA	Ferric enterobacter receptor
<i>oprQ</i>	PA2760	OprQ	Similar to Opr D named E3
<i>pilQ</i>	PA5040	PilQ	Type 4 fimbrial biogenesis protein Pil Q
<i>popD</i>	PA1709	PopD	Translocator protein
<i>opbA</i>	PA2291	OpbA	62 % similar to Opr B of PA
Gated porins			
<i>algE</i>	PA3544	AlgE	Alginate production protein Alg E
<i>fptA</i>	PA4221	FptA	Fe III-pyochelin receptor
<i>fpvA</i>	PA2398	FpvA	Ferripyoverdine receptor
<i>icmP</i>	PA4370	IcmP	Insulin-cleaving metalloproteinase ICHP
<i>cirA</i>	PA1922	CirA	56 % similarity to iron-regulated colicin and receptor of <i>E. coli</i>
<i>feeA</i>	PA3901	FecA	75 % similar to ferric citrate receptor of <i>E. coli</i>
<i>fiuA</i>	PA0470	FiuA	98 % similar to hydroxamate-type ferric siderophore receptor of <i>P. aeruginosa</i>
<i>hasR</i>	PA3408	HasR	62 % similar to haem acquisition protein HasR of <i>S. marcescens</i>
<i>hxC</i>	PA1302	HxC	57 % similarity of ton-dependent haem receptor Tdh A of <i>H. ducreyi</i>
<i>phuR</i>	PA4710	PhuR	Haem/haemoglobin uptake receptor PhuR
<i>optH</i>	PA4675	OptH	62 % similar to ferric aerobactin receptor IutA
<i>optI</i>	PA4897	OptI	52 % similar to OH haemin receptor of <i>P. aeruginosa</i>
<i>optO</i>	PA2335	OptO	37 % similar to pesticin, <i>Y. pestis</i>

(continued)

Table 2.4 (continued)

Gated porins			
<i>pptS</i>	PA2466	OptS	63 % similar to ferrioxamine receptor Fox A of <i>Y. enterocolitica</i>
<i>ufrA</i>	PA1910		99 % similar to undefined iron transport receptor
<i>popD</i>	PA1709		Translocator protein Pop D, Pep D
Efflux porins			
<i>czcC</i>	PA2522	CzcC	59 % similarity to Czc C of <i>R. eutropha</i>
<i>popN</i>	PA1698	PopN	Type III secretion protein
<i>pscC</i>	PA1716	PscC	Type III secretion protein
<i>xcpQ</i>	PA3105	XcpQ	General secretion pathway protein D
<i>xcpW</i>	PA3100	XcpU	General secretion pathway protein H Pil D-dependent protein Pdd B
<i>xqhA</i>	PA1868	XqhA	Secretion protein Xqh A
<i>hxcQ</i>	PA0685	XcpQ	Secretion protein type II
<i>oprM</i> , formerly <i>oprK</i>	PA0427	OprM	Outer membrane component of a multidrug efflux system (Dn stream, <i>mexA</i>)
<i>oprN</i>	PA2495	OprN	Multidrug efflux protein OprN
<i>oprJ</i>	PA4597	OprJ	Multi drug Efflux system (Dn stream <i>mexC</i> and <i>mexD</i>)

The consequence of the poor permeability is that many substrates utilize other pathways to cross the outer membrane to reach the desired concentration. OprF is the major channel for larger substrates and can be considered a general or non-specific water-filled channel (Sugawara et al. 1996; Tamber et al. 2006). The porins are broadly classified into four groups:

- General porins*: They allow the passage of a wide range of diverse compounds into the cell.
- Specific porins*: They are stereo specific and help in uptake of specific substances by binding sites.
- Gated porins*: They selectively take up large molecules such as iron-siderophore complexes.
- Efflux porins*: These channel tunnels help to remove toxic molecules from the cell in association with cytoplasmic membrane and periplasmic linker proteins.

2.2.3.1 General Porins

The hydrophilic compounds transverse the outer membrane through these non-specific porins. Transport is passive and depends on the physicochemical properties of the solute. The size of the molecule that can pass through these porins depends upon the diameter of these water-filled porins. Porins OmpF and OmpC of *E. coli* allow diffusion of molecules up to 600 Da. The external vestibules of

non-specific porin channels are rich in charged amino acids, and there are charges also around the restriction zone. General porins support the influx of solutes in nutrient-rich medium which can be metabolized by enzymes in the periplasm or transported to the cytoplasm via high-affinity cytoplasmic membrane transporters. In general outer membranes of pseudomonads show low permeability.

Porins of *P. aeruginosa* have been subject of extensive studies, and two types of porins OprD and OprF have been identified to permit passage of general substrates. OprD is a specific porin and allows conduction of small molecules, generally less than 200 Da. OprF is a member of OmpA family of outer membrane proteins and is responsible for the permeation of molecules between 200 and 3000 Da (Bellido et al. 1991). Functional heterogeneity and lack of homology of OprF to non-specific general porin family as well as limited expression of outer proteins in *Pseudomonas* are considered to contribute to high resistance towards many toxic compounds. The disaccharide carbohydrates appear to be transported through OprB porins.

OprF Porins OprF is the major outer membrane protein of *P. aeruginosa* with multiple functions as required for cell growth in low-osmolarity medium and for cell shape and has a role in antimicrobial drug resistance. It functions as a non-specific porin. In *P. fluorescens* it plays an important role in root adhesion for root colonization (Sugawara et al. 2006). In structure and function, OprF resembles porin, OmpA of *E. coli* and, possibly, a trimer that is associated with both LPS and peptidoglycan. The three domains of the OprF are:

1. The N-terminus of ~160 amino acids containing eight antiparallel sheets to form β -barrel structure.
2. A loop or hinge region of 161–209 amino acids containing a poly-proline–alanine repeat region and two disulphide bonds.
3. The C-terminal domain of 210–326 amino acids of OprF that is highly conserved with OmpA family proteins, shows 56 % similarity with C-terminal domain of OmpA. The C-terminal domain is a globular domain and forms a non-covalent bond with peptidoglycan in the periplasm. The C-terminal region is linked to N-terminal domain by a proline-rich hinge and loop region containing two disulphide bonds. The disulphide bonds are not common to all pseudomonads.

Porin function analysis by liposome swelling and planar lipid bilayer studies showed that *P. aeruginosa* OprF is a non-specific, weakly selective channel with one of the two channel sizes. These channels can either be small (0.36 nS) or large (2–5 nS). Large channel appears to be contradictory to low permeability of the outer membrane of *Pseudomonas*. But studies have shown that it is responsible for large exclusion limits. Only about 400 out of 200,000 OprF channels are large channels. Full-length OprF protein is required for large pore formation, while mutants truncated with C-terminal form only smaller-sized pores. Similar observations have been reported in *P. fluorescens* OprF. These are antigenic in nature and a portion of OprF developed as vaccine for *P. aeruginosa* infection (Gilleland et al. 2000; Larbig et al. 2001). The expression of porin OprF is substantially

down regulated in lungs of patients suffering from cystic fibrosis. Both OprF and OprF antibodies have been isolated from mucus of chronic patients. Expression of OprF is under the control of AlgU regulator which also regulates alginate production and mucoidy in *P. aeruginosa* as well as the ECF sigma factor SigX. Additionally, the OprF proteins of *P. aeruginosa* and *P. fluorescens* are involved in adherence to surface receptors in their respective hosts. The OprF homologue in *P. fluorescens* is a fibronectin-binding protein (Krishnan and Prasadarao 2012).

OprG Porin *Pseudomonas aeruginosa* lack the general porins such as OmpF of *E. coli* and other gram-negative bacteria which allow diffusion of small hydrophilic compounds. This makes *P. aeruginosa* highly impermeable and resistant to antibiotics. On the contrary OprG is a major OM protein in *P. aeruginosa* which is a member of OmpW family of OM proteins widespread in gram-negative bacteria with orthologs found in different classes of Proteobacteria. Expression of OprG is dependent on growth conditions, suggesting a complex regulation. Decreased expression of OprG has been linked to increased resistance to norfloxacin, tetracycline and kanamycin (McPhee et al. 2003; Peng et al. 2005). Increased expression of OprG was reported under high-iron conditions when grown under anaerobic conditions. However, knocking out of gene *oprG* did not corroborate these observations (McPhee et al. 2009). OprG from *P. putida* having similarity of 70 % with OprG of *P. aeruginosa* has a high emulsifying activity suggesting its involvement in uptake and utilization of hydrocarbons.

However, the X-ray crystal indicates that OprG of *P. aeruginosa* is a hydrophobic channel comprising of right standard barrel leading from the extracellular surface to a lateral opening in the barrel wall. The OprG barrel is closed from the periplasm by interacting polar and charged residues on opposite sides of the barrel wall. Crystal structure and the biochemical data suggest that OprG mediates the diffusion of small hydrophobic molecules across the OM by a lateral diffusion mechanism similar to that of *E. coli* FadL (Touw et al. 2010).

The distinctive feature of OprG is that the lumen of the barrel on the extracellular side is lined by hydrophobic residues that form a binding site for hydrophobic molecules.

2.2.3.2 Specific Porins

Pseudomonads use specific porins for the uptake of small molecules. This is in sharp contrast to other gram-negative bacteria which use specific porins for uptake of large and bulky substrates such as maltodextrins and nucleotides. There are a large number of specific *Pseudomonas* porins that have many sequence related proteins (paralogues) in the same organism. These are unique to pseudomonads.

Specific porins have saturated stereospecific binding sites for their substrates and follow Michaelis–Menten kinetics. Therefore, rate of uptake is accelerated at low substrate concentrations and plateaus when the sufficient substrate is present to saturate the binding sites (Nikaido and Vaara 1985). The presence of specific porins provides competitive advantage to the organisms in nutrient deficient

environments. The specific porins in pseudomonads enable the cells to uptake the oligotrophic levels of structurally diverse compounds and thus complement the low uptake activity of OprF general porins. *Pseudomonas* has three different types of porins, OprP, OprB and OprD.

Specific porins are rich in charged amino acids and share several conserved features leading to similar tertiary structures. There are several regions that have 12–25 alternating polar and non-polar amino acids flanked with aromatic residues but no stretches of hydrophobic residues. These regions correspond to the β -strands and form walls of channels. The aromatic amino acids help anchor porins into membranes. The β -strands form long loops at the extracellular surface which are highly variable. These loops help in stabilizing the porins by interacting with LPS and other porin monomers. The C-terminal region is the most conserved region and generally the aromatic amino acid is the terminal amino acid. These shared features of specific porins of *Pseudomonas* indicate that their tertiary structure is conserved (Koebnik et al. 2000).

Pseudomonas porins are composed of three subunits, and each subunit comprises of a barrel made of 16 β -strands. This is similar to general porins of gram-negative bacteria but differs from Lam β porin of *E. coli* that has been extensively studied and comprises of 18 β -strands per monomer. Substrate binds to the extracellular long loop and the barrel walls contain residues that facilitate passage of the substrate.

OprP/OprO Porins OprP is phosphate-specific porin induced under phosphate-limiting conditions (i.e. <0.15 mM) by PhoB regulator which also controls the expression of periplasmic phosphate-binding protein. OprP protein has a molecular weight of 48,000 Da. Purified OprP channel contains a binding site for phosphate. The channel is permeable to small anions, and it is blocked by the binding of phosphate to its binding site. Molecular architecture has been predicted to consist of 16-stranded β -barrel per monomer. By site-directed mutagenesis, it was established that lysine-121 in the loop 3 region was part of the phosphate-binding site. Lysine-121 binding site has the highest binding affinity in OprP but still is of lower affinity than the periplasmic phosphate-binding protein; thus, the phosphate will flow along the concentration gradient to the periplasm. Lysine residues at position 74 and 126 probably represent secondary non-rate-limiting binding sites (Hancock and Worobec 1998; Hancock and Brinkman 2002).

The *OprO* gene lies upstream of *OprP* gene in *P. aeruginosa* and the two proteins O and P share 76 % similarity in amino acid composition. Like OprP, the OprO is also inducible under phosphate starvation conditions but is produced after the cells enter the stationary phase. However, OprO preferentially binds pyrophosphate. *Pseudomonas fluorescens* produce an outer homologue of P protein inducible by PO₄ starvation named Ag1. This is not homologous to any other protein and measures 55 kDa as compared to OprP 48 kDa (Leopold et al. 1997).

Opr D Family Porins OprD has been studied extensively as it plays an important role in antibiotic resistance in *P. aeruginosa* particularly broad spectrum β -lactam antibiotic, imipenem. Opr D is a specific porin that binds basic amino acids, lysine,

arginine and dipeptides, containing basic amino acid residues and imipenem and related antibiotics. OprD also acts as a general porin allowing the passage of unrelated small molecules such as gluconate.

The OprD porin family of outer membranes of *P. aeruginosa* required a carboxylic acid group for efficient transport. These have been renamed as outer membrane carboxylate channels (Occ channels) and are divided into two subfamilies OccD and OccK on the basis of their different substrate specificities.

OccD family members transport positively charged amino acids, and OccK proteins have preference for cyclic compounds with a net negative charge such as benzoate. The basic requirement is that they possess a carboxylic group. Thus, they take up a variety of compounds while maintaining an effective OM barrier (Table 2.5).

Table 2.5 List of Specific porins of the OprD family of *Pseudomonas aeruginosa* (www.pseudomonas.com; www.crudr.ubc.ca/bobh/omps)

Gene name	PA number	From nucleotides	To nucleotides	Similarity to other proteins	# of A.A.s
<i>opbA</i>	PA2291	2522616	2521258	62 % similar to OprB of <i>P. aeruginosa</i>	452
<i>opdB</i>	PA2700	3053843	3055150	57 % similar to OprD	435
<i>opdC</i>	PA0162	184594	185928	58 % similar to OprD	444
<i>opdD</i>	PA1025	1110947	1112197	62 % similar to PhaK of <i>P. putida</i> ; OprD family	416
<i>opdF</i>	PA0240	271838	270573	53 % similar to OprE; OprD family	421
<i>opdG</i>	PA2213	2432312	2433562	60 % similar to PhaK of <i>P. putida</i> ; OprD family	416
<i>opdH</i>	PA0755	824198	822915	58 % similar to OprE; OprD family	427
<i>opdI</i>	PA0189	216908	215550	55 % similar to OprD	452
<i>opdJ</i>	PA2420	2702925	2704343	51 % similar to OprD	472
<i>opdK</i>	PA4898	5495712	5494459	56 % similar to PhaK of <i>P. putida</i> ; OprD family	417
<i>opdL</i>	PA4137	4626661	4627917	69 % similar to PhaK of <i>P. putida</i> ; OprD family	418
<i>opdN</i>	PA4179	4674943	4676238	58 % similar to PhaK of <i>P. putida</i> ; OprD family	431
<i>opdO</i>	PA2113	2324783	2323554	62 % similar to PhaK of <i>P. putida</i> ; OprD family	409
<i>opdP</i>	PA4501	5038900	5040354	52 % similar to OprD	484
<i>opdQ</i>	PA3038	3400683	3401948	65 % similar to PhaK of <i>P. putida</i> ; OprD family	421
<i>opdR</i>	PA3588	4021918	4020668	56 % similar to OprE; OprD family	416
<i>opdT</i>	PA2505	2823919	2822573	57 % similar to OprD, named OprD3 in Gene bank	448

The substrate-specific channels differ from the porins in that porins are smaller and allow diffusion of only a limited number of compounds (Nikaido 2003). Furthermore, the substrate-specific channels show higher binding affinity with their substrates as compared to porins. Such channels mediate uptake of water soluble and low molecular weight compounds like antibiotics by passive diffusion. Though they are present in all gram-negative bacteria, they are of special significance in *Pseudomonas*, because due to their ubiquitous nature, they are able to thrive in nutrient-poor environment (Eren et al. 2012, 2013).

Pseudomonas aeruginosa have about 30 substrate-specific channels, 19 of which are Occ responsible for uptake of majority of small molecules. Drug resistance is also attributed to lack of large channel porins such as OmpF/i of *E. coli*. OprD is similar to *E. coli* homologue OmpF with 15 % amino acid resemblance. OprD monomer consists of 16-stranded β -barrel. Loop 2 and loop 3 are involved in the binding of substrate, and the deletion mutants in these regions result in imipenem susceptibility. Deletion mutations in loops 5, 7 and 8 result in large channels and permit the passage of multiple antibiotics (Huang and Hancock 1996; Nikaido and Takatsuka 2009).

OprD is moderately expressed and regulated by multiple systems. It is induced by ArgR regulator; besides this alanine and glutamate may induce OprD independent of ArgR. OprD synthesis is also repressed by MexT (which also induces Mex EF–Opr N efflux system), salicylate and catabolite repression mechanism and is activated by arginine/ArgR and a number of other amino acids as carbon and nitrogen sources (Ochs et al. 1999).

Genome sequence showed that OprD is a part of a large family of porins. Nineteen OprD homologues have been identified in *P. aeruginosa* which show 46–57 % similarity in amino acid composition. Many homologues of OprD in *P. aeruginosa* play role in amino acid and peptide transport (Stover et al. 2000). *Pseudomonas putida*, *P. syringae* and *P. fluorescens* carry 21, 10 and 10 homologues of OprD porins, respectively (Nelson et al. 2002; Winsor et al. 2011). More number of OprD homologues is expected in pseudomonads and related bacteria. Phylogenetic analysis of *P. aeruginosa* porins revealed two subclasses, the OprD group and OprK group. Eight of these homologues were more closely related to Opr D and play a role in amino acid uptake. Other 11 are similar to the PhaK porin of *P. putida* which is required for growth on phenyl acetic acid (Olivera et al. 1998). This also includes OprE porin. The homologues facilitating diffusion of aromatic compounds have been identified in other pseudomonads. These are TbuX toluene channel in *Ralstonia picketti*; m-xylene channel (Xyl N) coded by TOL plasmid in *P. putida*; SalD, salicylate ester channel in *Acinetobacter*; and putative p-cymene channel, cymD of *P. putida*, and cumene channel, cumH of *P. fluorescens* (Schulz 2002).

OprE level was found to be decreased in *P. aeruginosa* mutants resistant to cephalosporin. Cloning and sequence analysis has shown that this is a homologue of OprD. OprE was induced in *P. aeruginosa* when grown anaerobically with NO_3^- as an electron acceptor. *Pseudomonas aeruginosa* also produce protein E₂, and its

level was found to be decreased in mutants showing increased resistance to cephalosporins and fluoroquinolones.

OprB Porins OprB porins of *P. aeruginosa* earlier referred to as OprD are closest homologues of LamB porins of *E. coli* with which it shares structural features like 18 β -strands. Sugars glucose, xylose, mannitol, fructose and glycerol enter through OprB porins. These porins are also present in *P. putida*, *P. fluorescens*, *P. chlororaphis* and *P. syringae* and facilitate uptake of glucose in first three organisms. For other carbohydrates their specificity is variable. OprB is induced by growth in minimal medium with glucose as the available carbon source and succinate acts as a catabolite repressor. β -Sheet content of these porins varies from 31 to 50 % indicating structural differences in these channels (Wylie and Worobec 1995).

Pseudomonas aeruginosa and *P. putida* have two OprB paralogues and the *oprB* gene lies in an operon of genes encoding homologues of porins having high affinity for glucose uptake system. Genes for the newly discovered *OprB* homologues (*OprA* in *P. aeruginosa* and *OprB2* in *P. putida*) lie downstream of glucose dehydrogenase gene. Enzyme glucose dehydrogenase plays a role in the low-affinity uptake pathway for glucose. *OprB* is positively regulated by glucose (Adewoye and Worobec 1999, 2000).

2.2.3.3 Gated Porins

Iron is an essential requirement for a number of proteins in aerobic respiration and *Pseudomonas* belongs to this category of bacteria. Usually iron is limiting in the environment because of its low solubility and high competition from other organisms. *Pseudomonas* produce siderophores which have high affinity for iron and also bind to specific surface receptors/uptake porins. *Pseudomonas* can also uptake iron from siderophores produced by other bacteria and fungi. *Pseudomonas aeruginosa* can acquire iron directly from haem and haem-containing proteins such as haemoglobin as well as *E. coli* siderophore enterobactin (Poole and McKay 2003).

Siderophore–iron complexes enter the cell through specialized receptor/porins called gated porins (IROMPs—iron repressible outer membrane proteins). The gated porins comprise a large family of 35 homologues in *P. aeruginosa*, 29 in *P. putida*, 23 in *P. syringae* and 26 in *P. fluorescens*, and many are expected in other *Pseudomonas* species (Cornelis and Matthijs 2002). They are also referred to as TonB-dependent receptors due to their dependence on a particular energy-transducing system. The diversity of gated porins in *Pseudomonas* indicates that each channel is specific for a particular siderophore.

Structural analysis of TonB-dependent receptors, FhuA and FecA, of *E. coli* shows these proteins are monomers of 22-stranded β -barrels. Near the N-terminus is a globular domain referred to as plug, containing four-stranded β -sheet and 4- α -helices that form part of the gated mechanism. Thus, plug undergoes conformational changes when the substrate binds the tonB, thereby leading to the opening

of the channel. Crystal structure studies show that for uptake the siderophore first docks into the binding pocket rich in aromatic residues on top of the plug domain. Then an arginine residue in the pocket shifts towards the substrate causing major conformational changes for uptake and transport of the substrate into the periplasm.

Pyoverdine Pyoverdine is a large family of siderophores and is a characteristic feature of fluorescent pseudomonads. Pyoverdine produced by *P. aeruginosa* is a dihydroxyquinoline containing fluorescent compound joined by a cyclic octapeptide. Pyoverdine has high affinity for ferric ion. This is predominant siderophore of fluorescent pseudomonad and is responsible for green fluorescence in these bacteria. There is considerable heterogeneity and specificity in siderophores produced by *Pseudomonas*. The specificity is mediated at the level of outer membrane receptors/gated porins, and FpvA is the receptor for type I pyoverdine of *P. aeruginosa* and has been extensively studied. Alignment of FpvA amino acid sequence against TonB-dependent receptors of other gram-negative bacteria predicts that FpvA monomer has 26 β -strands. Two residues in 6th loop, Y₃₅₀ and A₄₀₂, have been implicated in the binding and uptake of ferric pyoverdine. Fpv A has a 70-amino-acid extension at the N-terminal and this is responsible for the induction of its expression as well as pyoverdine biosynthetic operon. The mode of action of FpvA differs significantly from the Ton B-dependent receptors of gram-negative bacteria. In the resting state, FpvA is bound to iron-free pyoverdine. This is then displaced with ferric pyoverdine, which then enters the cell (Shen et al. 2002).

PupA and PupB are the ferric pyoverdine receptors of *P. putida*. They are also referred to as pseudobactins in this species. These are homologous to Fpv A and other Ton B receptors of *P. aeruginosa*. The Pup A is a specific receptor for ferric pseudobactin 358 and PupB facilitates transport via two siderophores, pseudobactin BN7 and BN8. Genome sequence analysis and specific PCR experiments indicate that *P. putida* contains multiple ferric pseudobactin (pyoverdines) receptors. The pyoverdine receptors, Pup A and Pup B of *P. putida* and Pbu A of *P. fluorescens*, have been cloned and characterized (Koster et al. 1993; Morris et al. 1994).

FptA/PfeA/Haem A number of secondary siderophores are produced by *Pseudomonas* that have lower affinity for iron than pyoverdine. Pyochelins produced by *P. aeruginosa* and *P. fluorescens* are distinct in the sense that it neither possesses hydroxamate nor catecholate chelating groups and binds to iron, cobalt and molybdenum. This siderophore is taken up by FptA receptor. In *P. aeruginosa*, pyochelin is involved in the acquisition of iron from transferrin and FptA is involved in virulence (Castignetti 1997). Another siderophore quinolobactin produced by *P. fluorescens* is taken up by 75 kDa outer membrane protein inducible by quinolobactin and repressed by pyoverdine.

Besides their own siderophores, *Pseudomonas* are known to use siderophores produced by different strains and species of pseudomonas, other bacteria and fungi. Each of the siderophores to be taken up induces the specific receptor and provides competitive advantages to *Pseudomonas* in the environment. PfeA receptor in *P. aeruginosa* shares 60 % similarity with *E. coli* enterobactin receptor; thus,

P. aeruginosa can take up enterobactin, the *E. coli* siderophore. Another protein PirA has been identified in *P. aeruginosa* which shares 72 % similarity with PfeA. Besides these several paired membranes of TonB family receptors are known in *P. aeruginosa*, *P. putida* and *P. fluorescens* that exhibit more than 50 % sequence similarity to each other. This redundancy may be responsible for the overall flexibility of these organisms (Dean and Poole 1993; Poole and McKay 2003).

Pseudomonas aeruginosa can also directly acquire iron from haem-containing proteins such as haemoglobin via two outer membrane receptors, HasR and PhuR. Both these systems help to support growth on haemin or haemoglobin as sole source of ferric ion. Genome analysis has shown that HasR has two homologues, OptI of *P. aeruginosa* with 52 % similarity and another with 57 % similarity with haem-utilizing protein of *H. influenza*. PhuR homologues are found in *P. putida*, *P. fluorescens* and *P. syringae*.

OprC is an unusual TonB-dependent receptor protein in *P. aeruginosa* that is specific for Cu^{++} . OprC is 65 % homologue with *P. stutzeri* NosA, an outer membrane porin required for the production of Cu^{++} containing nitrate reductase. Opr C is synthesized anaerobically and is repressed in high Cu^{++} concentration in the medium and shows substantial homology to PfeA (Hancock and Brinkman 2002). OprH is an outer membrane protein that is upregulated upon Mg^{++} starvation in PhoPQ two-component regulatory system for *OprH*, *PhoP* and *PhoQ* operon. OprH forms an eight-stranded β -barrel as a gated porin for divalent cation (Macfarlane et al. 1999). Another unusual ton B-dependent receptor protein in *P. aeruginosa* is PA1271, a homologue of BtuB, the vitamin B12 uptake porin.

2.2.3.4 Efflux Porins

P. aeruginosa is known for its intrinsic multiple antibiotic resistance. This resistance has been linked to overexpression of any of the three effluxes, operons (Poole 2001). Amongst these resistance-nodulation-cell division (RND) family is the most important. This involves a three-component efflux pathway, comprising of (1) cytoplasmic membrane pump protein, (2) a peripheral cytoplasmic membrane linker (membrane fusion protein) and (3) an elaborate outer membrane/periplasmic channel protein. These proteins are highly conserved. The outer membrane channel proteins (efflux porins) TolC of *E. coli* are the best studied example of channel tunnel. Besides being a multiple antibiotic efflux protein, it is also a component of type I secretion system or haemolysin (Koronakis et al. 1997; Hancock 1997).

RND in *Pseudomonas aeruginosa* plays an important role in producing multi-drug resistance and comprises of efflux pump MexB which associates with two other classes of proteins, the outer membrane channel, OprM belonging to the OMF (outer membrane factor) family of proteins and the periplasmic “adapter” protein such as MexA, classified as MFP (membrane fusion protein) family. All the three components are essential for the drug efflux (Nikaido and Takatsuka 2009; Zavascki et al. 2010).

P. aeruginosa has 18 outer membrane proteins, 11 of which fall into one phylogenetic subclass and are involved in multiple antibiotic efflux system.

Table 2.6 Efflux porins of the OprM family of *Pseudomonas aeruginosa* (<http://www.cmdr.ubc.ca/bobh/omptotal.html>)

<i>opmA</i>	PA2837	OpmA	53 % similar OprN
<i>opmB</i>	PA2525	OpmB	50 % similar OprH
<i>opmD</i>	PA4208	OpmD	56 % similar OprN
<i>opmE</i>	PA3521	OpmE	52 % similar OprN
<i>opmF</i>	PA4592	OpmF	40 % similar type I secretion protein Cya E of <i>B. pertussis</i> , OprM family
<i>opmG</i>	PA5158	OpmG	53 % similar to aromatic efflux pump of <i>S. aromaticivorans</i> OprM
<i>opmH</i>	PA4974	OpmH	54 % similar to efflux porin Tol C of <i>E. coli</i>
<i>opmI</i>	PA3894	OpmI	51 % (as in OpmG)
<i>opmJ</i>	PA1238	OpmJ	51 % similar OprN
<i>opmK</i>	PA4144	OpmK	49 % similar CyaE of <i>B. pertussis</i> OprM
<i>opmL</i>	PA1875	OpmL	40 % similar AprF
<i>opmM</i>	PA3404	OpmM	68 % similar AprF
<i>opmQ</i>	PA2391	OpmQ	48 % similar OprM

Important amongst these are OprM, OprN and OprJ. Amongst the other seven, CzcC is involved in cation efflux of the detoxification mechanism, AprF is involved in type I secretion of alkaline protease, and the third, the OpmH is very close to *E. coli* TolC, with 54 % similarity. The other four, OpmF, OpmK, OpmL and OpmM, are similar to CyaE of *Bordetella pertussis* or to AprF and may be a component of type I protein secretion pathway. The efflux porin, TolC, can serve more than one secretion/efflux system (Koronakis et al. 1997, 2004).

OprM OprM is the major outer membrane efflux porin of *P. aeruginosa* involved in intrinsic multiple antibiotic resistance (Table 2.6). A mutation in OprM leads to 10–1000 times increase in sensitivity to many antibiotics and cloning of OprM gene complements for such deletions. Mutation in *nalB* (*mexR*) gene results in overexpression of OprM and its neighbouring linker and pump proteins, MexA and MexB, leading to resistance to broad range of antibiotics such as fluoroquinolones, tetracycline, chloramphenicol, macrolides, trimethoprim and β -lactams. OprM shares 21 % similarity with TolC. OprM is a trimer comprising of a single channel tunnel spanning the outer membrane and periplasm. This forms a 12-stranded β -barrel (4 β -strands for each monomer) that lodges in the outer membrane and sits atop coiled 12-helix α -helical barrel that spans the periplasm and is in contact with MexB pump/MexA linker complex in the cytoplasmic membrane (Wong et al. 2001). Mutagenesis by additions/deletions in the loop region of the outer barrel does not affect function, but they are not permissible within the α -helical barrel. Yoshihara et al. (2002) have suggested that OprM channel, a component of outer membrane multidrug efflux pump, works as a gated channel.

OprJ and OprN OprJ and OprN are other two efflux porins of *P. aeruginosa* that are components of efflux pump systems MexCD–OprJ and MexEF–OprN which

are homologous to MexAB–OprM. These porins are normally silent and expressed due to mutation as part of *MexCD–OprJ* and *Mex EF–OprN* operons leading to multiple drug resistance. Most overexpressed mutants are in *nfxB* repressor and *mexT* (*nfx C*) activator genes. Mutation in *mexT* leads to coordinated upregulation of Mex EF–Opr N efflux system and down regulation of OprD (Kohler et al. 1997; Li and Poole 2001; Nikaido and Pages 2012).

Pseudomonas putida has several homologous systems with the ability to efflux aromatic hydrocarbons. The efflux porins ArpC, MepC, TtgC and TtgI influence antibiotic susceptibility when overexpressed. Porins, OpmG, OpmH and OpmI are linked to aminoglycoside efflux in *P. aeruginosa* (Poole 2001).

AprF and Protein Secretion *Pseudomonas aeruginosa* secretes many proteins involved in virulence and other biological functions by using secretion system type II. The alkaline protease, coded by *aprA* gene, is secreted by the three-component type I secretion system, AprDEF, where AprF is the outer membrane component. AprF functions in the manner similar to TolC. Besides this there are five other homologues that could be engaged in type I secretion. The type II and type III protein secretion pathways in *P. aeruginosa* utilize outer membrane proteins that form ringlike structures with multiple subunits (Tomassen et al. 1992; Brok et al. 1999). These form channels for secretion of proteins and are probably gated porins as large channels will compromise low permeability of the outer membrane. Type I general secretion pathway XcpQ and its homologue XqhA has been recognized in *P. aeruginosa*. For type III secretion system XcpQ, homologue PscC acts as a channel.

2.2.4 Outer Membrane Vesicles

Membrane vesicles first reported in 1989 have dynamic features and are constantly discharged from the surface of gram-negative bacteria during growth (Mayrand and Grenier 1989). These are extruded from the surface and entrap some of the periplasm (Fig. 2.5). They possess outer membrane proteins (OMPs), LPS, phospholipids and periplasmic constituents in a manner similar to the bacterium but on a much smaller scale. These measure 50–250 nm in diameter and are spherical bound by trilamellar membrane structures, similar to outer membrane structure, and are released from the surface of gram-negative bacteria (Kadurugamuwa and Beveridge 1997; Zhou et al. 1998; Beveridge 1999; Mashburn and Whiteley 2005). They form blebs from the outer membrane and encapsulate periplasm. OMVs of *P. aeruginosa* were found to contain protease, phospholipase C, alkaline phosphatase and an autolysin (Li et al. 1998; Kulp and Kuehn 2010). These OMVs also contained proelastase and was the evidence of their periplasmic encapsulation since the pro-amino-acid sequence is cleared from enzyme only after translocation across the outer membrane. The OMVs are formed with the abrupt change of normal low curvature to high curvature to form vesicles (Kadurugamuwa and Beveridge 1995, 1996).

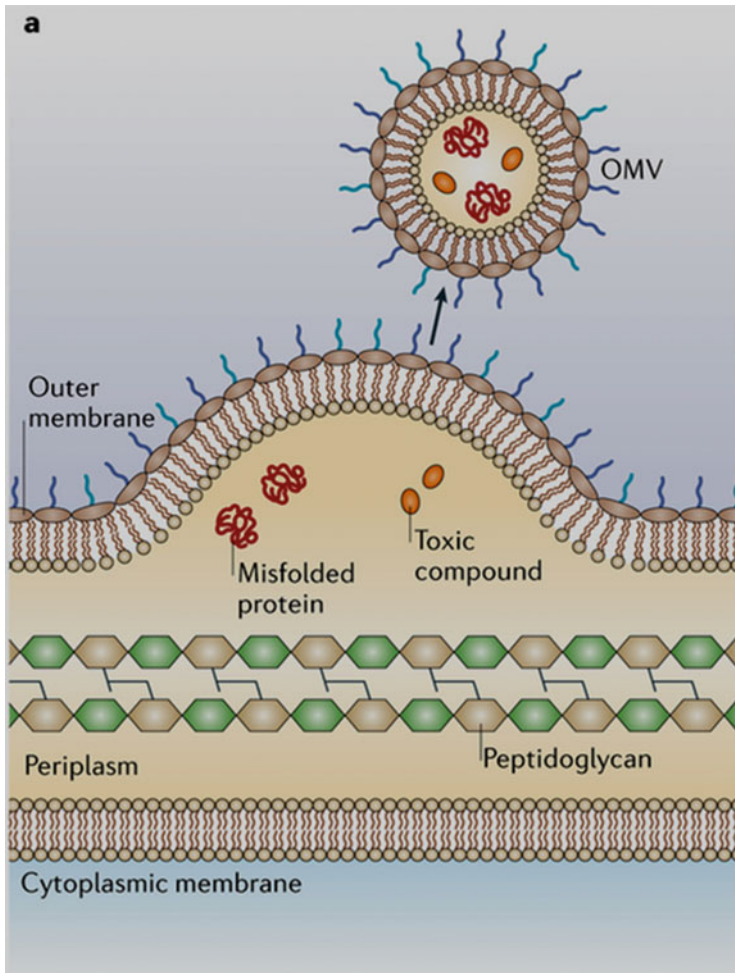


Fig. 2.5 Formation and release of outer membrane vesicle (Schwechheimer and Kuehn 2015)

With LPS, phospholipids and OMPs still integral constituents of the OMV bilayer and outer membrane structure are retained. One important variation observed is that of the absence of A-band LPS (CPA), and only B-band LPS (OSA) is present in OMVs of *P. aeruginosa*. Maybe this pooling of B-band forces the outer membrane into high-curvature structures bending to the formation of blebs and release of OMVs.

Periplasm of gram-negative bacteria also contains autolysins which are involved in peptidoglycan synthesis along with penicillin-binding proteins. *Pseudomonas aeruginosa* produces 11 different types of autolysins, and one of these, 26 kDa peptidoglycan hydrolase (PGase), is packed in OMVs (Li et al. 1998; Bauman and Kuehn 2006). OMVs can lyse other bacteria as long as these bacteria are suffering

from low nutrition and growing poorly. OMV-mediated lysis does not occur in actively growing cells. OMVs lyse surrounding foreign bacteria thereby liberating complex nutritious substances for the donor cells. OMVs would not kill neighbouring cells of the identical strains as the OMV PGase is one of their normal autolysin and regulated as such, thus benefiting the population of a single or identical clone.

A number of gram-negative bacteria induce pathogenesis by production of virulence factors such as haemolysin, aerolysin, neurotoxin, etc. The factors diffuse into the tissue and break down the cells. These factors along with certain enzymes, e.g. phospholipase C, proteases and proelastase, are packaged into OMVs and are protected from the inactivating enzymes of the host environment (Wai et al. 1995). OMVs of *P. aeruginosa* have been reported to be packaged with a number of virulence factors, viz. phospholipase C, haemolysin, alkaline phosphatase, cystic fibrosis transmembrane regulator (CFTR) inhibitory factor, multifunctional *Pseudomonas* quinolone signaling (PQS) molecule 2-heptyl-3-hydroxy-4-quinolone, murein hydrolases, protease and β -lactamase, to be released at the site of infection (Ciofu et al. 2000; Mashburn and Whiteley 2005; MacEachran et al. 2007; Bomberger et al. 2009). Plant pathogen, *Xanthomonas campestris*, also generates membrane vesicles packaged with cellulose, β -glucosidase and type 3 secretion system (Sidhu et al. 2008). Biochemical analysis of purified OMVs on the basis of density gradient showed that OMVs consisted of proteins and lipids of the outer membrane and periplasm and did not contain any inner membrane and cytoplasmic components (McBroom and Kuehn 2007).

Proteomic analysis of OMVs from different gram-negative bacteria indicated the presence of more than 200 vesicular proteins. Several of these were found to be common in OMVs derived from several species of gram-negative bacteria and grouped as (1) outer membrane porins OMPs, PorA, PorB and OprF; (2) murein hydrolases; (3) multidrug efflux pumps known to release toxic compounds, e.g. Mtr, Mex and TOIC; (4) ABC transporters; (5) protease/chaperone proteins; (6) the motility proteins related to fimbriae/pili; and (7) various virulence factors, e.g. haemolysin, IgA, protease, etc. (Kulp and Kuehn 2010). OMVs of *Pseudomonas aeruginosa* have also been reported to carry DNA, the DNA-binding proteins such as DNA-binding stress proteins and DNA methyl-transferase and host of other proteins. A total of 338 proteins, associated with outer membrane, have been identified by Choi and his group (Choi et al. 2011; Maredia et al. 2012). *Pseudomonas aeruginosa* associated with lung infections such as cystic fibrosis, chronic obstructive pulmonary disease (COPD) and pneumonia produces OMVs carrying virulence factors, viz. phospholipase C, haemolysin, alkaline phosphatase, cystic fibrosis transmembrane regulator (CFTR) inhibitory factor (Cif), 2-heptyl-3-hydroxy-4-quinolone (PQS), murein hydrolase, protease and β -lactamase (Bauman and Kuehn 2006; Bomberger et al. 2009). Thus, OMVs serve as a secretion mechanism for virulence factors as well as general bacterial response to environmental stressors acting on the envelope. Exposure to environmental stressors results in increased production of OMVs as well as enhanced activity of AglU, the sigma factor that controls MucD expression (MacDonald and Kuehn 2013).

2.3 Periplasm

By definition the “periplasmic space” refers to the compartment bounded by the cytoplasmic membrane and the “molecular sieve” that has a number of physiological and biochemical functions (Mitchel 1961). The equivalence of the “molecular sieve” is the outer membrane of the gram-negative cell envelope. Thus, the periplasmic area and the peptidoglycan–lipoprotein complex occupy the same zone of the cell envelope, and one accommodates periplasmic components, whereas the other supports and reinforces the wall envelope. When the continuity of the outer membrane surrounding the periplasmic space is interrupted by changes in the outer membrane or in the LPS, the periplasmic enzymes, the binding proteins and pigments are released into the medium. Treatments such as change in ionic equilibrium, pH, temperature and mild detergents disrupt the outer cell membrane (Costerton et al. 1974). Exposure of cells of *P. aeruginosa* to 0.2 M Mg^{2+} or 20 % sucrose also modifies the cell wall to allow the inward passage of lysozyme and the release of periplasmic enzymes. The barrier layer of the outer membrane is also disturbed when cells are grown without an essential amino acid (Knox et al. 1966), at an elevated pH, or in the presence of polymyxin B, and when either protein or peptidoglycan synthesis is inhibited. Defects in the barrier layer of the outer cell wall may also be genetically determined, and “periplasmic leaky” strains have been described in *E. coli* and in *P. aeruginosa*, which release periplasmic enzymes into the medium during growth. In both of these strains (Huang et al. 2008), the periplasmic enzyme in question (alkaline phosphatase—APase) is released as an enzyme–LPS complex. Physiological studies have shown that guanosine 5'-triphosphate (GTP) is hydrolyzed by phosphatase in the periplasmic space without the molecule being present at any time in the cytoplasm, and direct localization of enzymes in the periplasmic area has been affected by reaction product deposition and by the use of ferritin-coupled antibodies. These enzymes are not exclusively periplasmic, but a large proportion of their molecules are found in this zone of the cell wall. The distribution of enzymes within the periplasmic zone of the cell wall has been examined (Wetzel et al. 1970). These enzymes are not firmly bound to structural wall components and are uniformly distributed throughout the periplasmic space (MacAlister et al. 1972) or may be bound to a variety of structures within the periplasm (Garrard 1972; Beveridge and Kadurugamuwa 1996).

The dynamic structure of the periplasm suggests that periplasm is the true compartment of the cell lying between the inner membrane (IM) and the outer membrane (OM). This is densely packed with proteins and is more viscous than cytoplasm (Mullineaux et al. 2006). It appears as a gel-like matrix containing thin layer of peptidoglycan and a variety of specialized proteins. The diameter of this region is more or less constant and measures 10–30 % volume of the cell. The specialized proteins present in the periplasm bind molecules such as sugars, amino acids, vitamins and ions. By association with other cytoplasmic membrane-bound structures, these proteins can release the bound compounds which are then transported to the cytoplasm. These proteins, referred to as chaperones, again fuse into the periplasm and bind another incoming molecule.

Table 2.7 Important group of enzymes present in periplasm and their functions

Enzyme type	Example	Function
Hydrolytic enzymes	Phosphatases	Degrading phosphate containing compounds
	Proteases	Degrading proteins and peptides
	Endonucleases	Degrade nucleic acids
Binding proteins	Sugar, amino acids, organic ions and vitamins	Binding substrate and docking with transport protein in membrane
Chemoreceptors	Chemotaxis	Sensing environment and changing cell behaviour in response
Detoxifying enzyme	β -lactamase	Degrading inactivation of β -lactam antibiotics

Thus, *Pseudomonas* have a typical diderm cell organization, and the periplasm is the storehouse for periplasmic enzymes, trafficking proteins, secreted material and newly synthesized outer membrane or peptidoglycan layer-directed components at some point during bacterium's metabolic life (Beveridge 1995, 1999).

The available evidence suggests that periplasmic enzymes are associated with LPS and other structural components of the cell envelope and that they are distributed throughout the periplasmic zone, without notable local concentration. The precise level at which these proteins are located within the periplasmic space is not yet defined, except in the cases of binding proteins, which are functionally associated with the outside of the cytoplasmic membrane, and to the APase of the marine *Pseudomonas* B16, which is bound to the components of the outer area of the periplasm.

The proteins present in periplasm are distinct from those present in the cytoplasm. Important groups of proteins other than peptidoglycan biosynthesis present in the periplasm are listed in Table 2.7.

The bacterium relies on environment signals to influence the synthesis and secretion pathways. Quorum sensing plays an important role and help coordinate response in bacterial population of biofilms. *Pseudomonas aeruginosa* implicated with cystic fibrosis requires induction before the virulence factors are synthesized. Even the osmotic differences between cytoplasm and periplasm may trigger the synthesis of membrane-derived oligosaccharides. The periplasm bound by double-layered membranes is always in dynamic flux state possessing an ever-changing variety of macromolecules reflecting the metabolic and environmental status.

Forsberg et al. (1970) examined the chemical nature of the structural components of the periplasmic zone of the marine *Pseudomonas* B16 and found that this material, known as “underlying soluble layer”, contained both protein and polysaccharide. A small molecular species ($<4 \times 10^6$ Da) was largely proteinaceous, whereas larger aggregates ($>4 \times 10^6$ Da) were composed of both protein and polysaccharide and were shown to be morphologically heterogeneous.

Periplasm is packed with proteins and it is more viscous than cytoplasm (Mullineaux et al. 2006). Cellular compartmentation allows gram-negative bacteria to sequester potentially harmful degradative enzymes such as RNase or alkaline

phosphatase. Other proteins that inhabit this compartment are periplasmic binding proteins which function in sugar and amino acid transport, chemotaxis and chaperone-like molecules that function in envelope biogenesis.

Proteomic analysis of *P. aeruginosa* using 2DE and MALDI-TOF/TOF system analysis identified 495 spots representing 395 proteins (Imperi et al. 2009). Majority of the proteins identified are involved in the carbohydrate and amino acid metabolism, and most of the abundant cytoplasmic proteins were chaperonins, ribosomal proteins and translation factors and those involved in transport, cell envelope integrity and protein-folding control. The cytoplasmic contaminants were identified as faint spots.

Genomic analysis predicted that 38 % of the *P. aeruginosa* PAO1 genome (~2123 ORF) codes for the proteins that are exported out of cytosol to cell envelope and to the medium. Most of these correspond to the environmental conditions or to the resistance pattern. Eleven proteins have been identified to be responsible for antibiotic resistance which included porins OprF, OprG, OprD, an outer membrane protein (OPM) and multidrug efflux pumps (Peng et al. 2005). Analysis of the proteins of the whole cell, membrane, periplasmic and extracellular proteins of *P. aeruginosa* strain isolated from CF-lung showed that proteins involved in resistance to antibiotics were upregulated during infection (Sriramulu et al. 2005; Godlewska et al. 2009).

2.4 Cell Wall

Cell wall comprising of peptidoglycan (murein) sacculus is nearly universally present in bacteria and lies in the periplasm, i.e. outside the cytoplasmic or the inner membrane (Rogers 1970; Nanninga 1998). Primary function of the cell wall is to preserve the integrity of the cell by withstanding the turgor pressure and maintain the definite cell shape during growth and cell division. The cell wall scaffold also holds other compounds of the cell envelope in position (Dramsı et al. 2008). Biosynthesis of cell wall is subject to inhibition by a number of antibiotics and such conditions will result in lysis of the cells during growth as is the case with lysozyme (Vollmer et al. 2008). In gram-negative bacteria, the cell wall sacculus is thin and lies in the periplasm, and generally it is single layered and is connected to the outer membrane by covalent and non-covalent interactions with proteins of outer membrane (Vollmer and Holtje 2004; Huang et al. 2008). This is in contrast to the gram-positive bacteria in which case the cell wall sacculus is multilayered and covalently attached to the cell surface proteins.

The murein sacculus of *Pseudomonas* resembles other gram-negative bacteria measuring 4 nm thick (Gan et al. 2008). The murein consists of glycan strands cross-linked by peptides forming three-dimensional network structure. Glycans comprise of *N*-acetyl-glucosamine (Glc NAC) and *N*-acetyl muramic acid (Mur NA) residues linked through β -1 $\rightarrow$ 4 linkage. The D-lactoyl group in the Mur NAC residue is substituted by a small peptide. In *Pseudomonas aeruginosa*, the penta-peptide is composed of L-alanine- γ -D-glutamate-meso-diaminopimelic acid-D-

alanine–D-alanine. The cell walls of *Pseudomonas* represent approximately 1 % of the cell dry weight and contain about 57–59 % proteins. The *Pseudomonas* cell walls differ from that of *E. coli* in having much smoother cell surface structure as the grains in *P. aeruginosa* are less than half (4.7 ± 0.6 nm) the size of *E. coli* coarse grains measuring approximately 10 nm in diameter (Vollmer 2008).

Treatment of sacculi of *P. aeruginosa* by trypsin and chymotrypsin removed the granules indicating their proteinaceous nature. In *E. coli* mucopeptide layer is linked to outer layer of the wall through lipoprotein which is removed by trypsin. In *P. aeruginosa*, though the covalently linked protein is less, it appears that lipoprotein is involved in the linkage between mucopeptide and the outer layer of the wall. Chemical analysis of sacculi of pseudomonads confirmed the presence of small amounts of proteins. Molar concentrations of major components, *N*-acetylglucosamine, *N*-acetylmuramic acid, glutamic acid, alanine and diaminopimelic acid, have been observed to be in the ratio of 1:1:1:1.73:1. Lysine was the other amino acid that was detected in cell walls of *Pseudomonas*, but its frequency was much less than diaminopimelic acid, i.e. one for every nine moles of diaminopimelic acid. In about 10 % cases, the diaminopimelic acid is covalently bound to protein through lysine (Heilmann 1974).

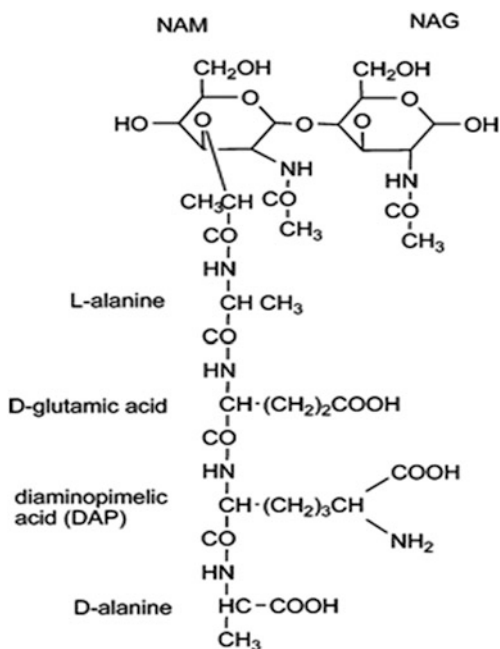
Cross-linking between free amino group of diaminopimelic acid and the terminal D-alanine is 25 % to 30 % depending upon the conditions of growth. Other gram-negative bacteria also show about the same level of cross-linking. Treatment of sacculi by EDTA or 8-hydroxyquinoline did not affect the shape or composition of the sacculus, but it was destroyed by lysozyme which hydrolyses the β -1,4-linkage between GlcNAC and MurNA. This indicates that the integrity of *N*-acetylglucosamine and *N*-acetylmuramic acid dimer is important in maintaining the structure and shape of the sacculi.

Isolated peptidoglycan structures (sacculi) maintain the structure of the cells from which they are isolated. Sacculi of *E. coli* are approximately 6 nm in thickness as determined by cryo-transmission electron microscopy of frozen-hydrated cell sections (Matias et al. 2003). Small-angle neutron-scattering analysis showed that 75–80 % of the surface of sacculus is 2.5 nm thick and the remaining 20–25 % may be up to 7 nm (Labischinski and Maidhof 1994).

2.4.1 Molecular Structure

The glycan moiety of the peptidoglycan is remarkably a uniform heteropolymer of β -1,4-linked alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid. The latter amino sugar, found only in bacteria and blue-green algae, was first discovered by Strange and Dark (1956). The 3-O-D-lactic acid ether of glucosamine in *P. aeruginosa* is substituted by the peptide of L-alanine–D-glutamic acid–meso-diaminopimelic acid and D-alanine–D-alanine (Fig 2.6). The last D-alanine is removed in the mature cell wall. The glycan reveals only few variations, such as acetylation or phosphorylation of the muramyl 6-hydroxyl groups and the occasional absence of a peptide or N-acetyl substituent. Variations have been noticed in

Fig. 2.6 Primary structure of the peptidoglycan unit of *Pseudomonas aeruginosa* comprising of *N*-acetyl-D-glucosamine- β -1,4 linked *N*-acetyl-muramic acid attached with tetrapeptide of L-alanine, D-glutamic acid, meso-diaminopimelic acid and D-alanine



the peptide stem, glycan strand and cross-linkage structure. Peptidoglycan variations have been related to phylogeny of bacteria (Schleifer and Kandler 1972; Heilmann 1974).

Glycan strands are formed by oligomerization of monomeric disaccharide peptide units by transglycosylation reactions. The average glycan chain length in gram-negative bacteria was estimated as 21 disaccharide units which is less than the one estimated from the fraction of 1,6-anhydro-MurNac residues to be 25–35 disaccharide units. In different pseudomonads, the glycan chain length varies between 10 and 65 disaccharide units in comparison to up to 30 in *E. coli* and 50–250 in case of *Bacillus* sp. (Quintela et al. 1995; Margolin 2009; Dmitriev et al. 2005).

Variations in peptidoglycan composition may involve amidation, hydroxylation, acetylation and attachment of amino acids and other groups at position 2 or 3 after the action of Mur ligase generally at the level of lipid II.

The peptide moiety is bound through its N-terminus to the carboxyl group of muramic acid and contains alternating L- and D-amino acids. The occurrence of amino acids with the D-configuration is a typical feature of the peptidoglycan. A fragment of the primary structure of a peptidoglycan is shown in Fig. 2.6.

Usually L-alanine is bound to muramic acid through its amino group followed by glutamic acid, which is linked by its γ -carboxyl group to an L-diamino acid, generally diaminopimelic acid in *Pseudomonas* and finally D-alanine–D-alanine dipeptide is attached to the diamino acid. In some cases the α -carboxyl group of glutamic acid is substituted and an additional D-alanine is found at the C-terminus. This part of the peptide moiety is called the peptide subunit. The free amino acid

group of the L-diaminopimelic acid, not bound to the peptide subunit, forms a peptide linkage to the C-terminal D-alanine of an adjacent peptide subunit or is substituted through an interpeptide bridge. Thus, the peptide moiety of the peptidoglycan can consist either of the peptide subunit or of the peptide subunit and an interpeptide bridge. The interpeptide bridges cross-link the peptide subunits and extend usually from the free amino group of the diaminopimelic acid of one peptide subunit to the D-alanine carboxyl group at position 4 of another peptide subunit. Rarely it may extend from the α -carboxyl group of D-glutamic acid to the carboxyl group of D-alanine of another peptide subunit. *Pseudomonas* show cross-linking between 25 and 30 % which is similar to other gram-negative bacteria. Cross-linking of the peptide stem provides rigidity to the cell and gram-positive bacteria show high degree of cross-linking (>70 %). The cross-linking reaction is catalysed by the transpeptidase domain of penicillin-binding protein. In gram-negative bacteria, cross-linking is mediated by a direct peptide bridge bond, while in gram-positive it is mediated through a peptide generally of 2–5 amino acids. The cross-linking may comprise of heptapeptide, L-ala–D-glut–meso-diaminopimelic acid–D-ala to diaminopimelic acid (D-ala-meso-diaminopimelic acid, or 3–4 bridges) or LD-diaminopimelic acid–diaminopimelic acid bridge (or 3–3 bridges). These are hexapeptide bridges, i.e. one shorter than heptapeptide. This is mediated by penicillin-insensitive LD-transpeptidase (Holtje 1998). The LD-diaminopimelic acid–diaminopimelic acid bridges account for 7–16 % total cross-linked mucopeptide (Glauner and Holtje 1990). Cross-linked trimers and tetramers have also been detected by HPLC. While the cross-linked dimers account for 30–40 %, the trimers are 3–4 % and tetramers 0.2 % of total mucopeptides (Glauner et al. 1988).

The peptidoglycan layer is assembled from components produced in the cytoplasmic membrane, and the two structures may be joined by nascent peptidoglycan. We must bear in mind that the turgor pressure of the living cell would force the cytoplasmic membrane outwards against the inelastic peptidoglycan layer. Thus, the peptidoglycan layer exerts morphological control over the cytoplasmic elements of the cell. The specific murein–lipoprotein called Lpp (Braun's lipoprotein), which is 12 to 14 nm long and composed of 57 amino acids, is covalently linked to the peptidoglycan of several enteric bacteria in such a way that it extends outwards towards the outer membrane (Braun and Wolff 1970; Braun 1975). Thus, OM is virtually stapled to peptidoglycan by lipoprotein Lpp. The lipid attached to it is embedded in OM. *E. coli* is reported to contain 50,000 molecules of Lpp per cell (Silhavy et al. 2010). In addition the proteins such as OmpA bind peptidoglycan non-covalently.

In *P. aeruginosa* three lipoproteins, Opr1, OprL and OprF, that connect the OM with the PG have been identified. OprF is a specific porin that allows passage of small molecules but is also associated with PG within the periplasm. In *Salmonella* spp. specific domains in the envelope have been recognized that promote interactions between outer membrane and PG and interactions between OMP and inner membrane proteins (IMP) involving PG (Deatherage et al. 2009). Such protein–protein interactions involving PG provides required strength to the cell envelope. The covalently linked lipid component of this molecule serves to anchor the outer membrane by hydrophobic interactions with the phospholipids of this

membrane-like layer. The lipoprotein of *E. coli* is free in the periplasm when it is first synthesized and that it is subsequently covalently bound to the peptidoglycan.

Variations in peptide stem arise either (1) due to the specificity of the Mur ligase and (2) those occurring in the later step. In *Pseudomonas* the first amino acid, L-alanine, is added by Mur C ligase, and the second amino acid, the D-glutamic acid, is added by Mur D ligase. The third amino acid is highly variable amongst bacteria, but in *Pseudomonas*, it is by and large only meso-diaminopimelic acid. The amino acids in position 4 and 5 are added as dipeptide D-alanyl-D-alanine.

The cell walls of *P. aeruginosa* are about half the thickness of that of *E. coli*. This means that even thin glycan is enough to maintain the shape and structure of the bacterial cell (Dmitriev et al. 2003).

2.4.2 Cell Wall Biosynthesis

Growth and expansion of the murein succuli is a prerequisite for cell growth and multiplication. Thus, synthesis of PG is of tremendous importance for growth and maintenance of cell. Furthermore, a number of antibiotics also inhibit cell wall synthesis. In gram-negative bacteria, PG synthesis takes place in two compartments: (1) cytoplasm in which the precursors are synthesized and (2) periplasm in which the precursors are ligated together to form a polymer. The process of complete synthesis of PG occurs in three steps (Fig. 2.7). First is the formation of activated nucleotide precursors, i.e. UDP-*N*-acetylglucosamine and UDP-*N*-acetylmuramyl pentapeptide (Barreteau et al. 2008). In the second step the precursors are assembled with undecaprenyl phosphate (lipid I) to form a lipid

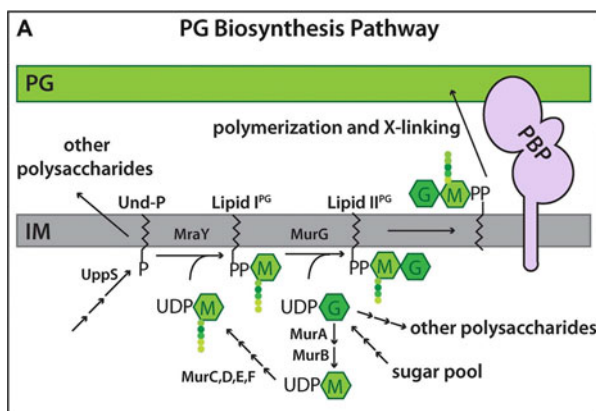


Fig. 2.7 Cell wall biosynthesis, dimer of MurNAc (M) and GlcNAc (G) and the attached pentapeptide, referred to as lipid II^{PG}, is synthesized in the cytoplasm. The lipid flips over through the cytoplasmic membrane and the dimer polymerizes with the existing peptidoglycan and the lipid moiety of lipid II is released. Lipid I^{PG} and lipid II^{PG} are undecaprenyl phosphate (C55-P) and undecaprenyl pyrophosphate (C55-PP), respectively (Paradis-Bleau et al. 2014)

anchored disaccharide–pentapeptide monomer subunit referred to as lipid II. While the first step takes place in the cytoplasm, the second occurs at the inner leaflet of cytoplasmic membrane (IM). The lipid II is flipped across the membrane by flippase, and the third step of polymerization of lipid II takes place in the periplasm. During this process the undecaprenyl pyrophosphate is released and the glycan chain is inserted into the sacculus (Rodríguez-Herva et al. 1996; Bouhss et al. 2008).

The enzymes and cofactors involved in the biosynthesis of PG are listed in Table 2.9. Key intermediates of precursor synthesis are nucleotide-activated amino sugars uridine diphosphate-*N*-acetyl-glucosamine (UDP-GlcNAc) and uridine diphosphate-*N*-acetylmuramic acid (UDP-MurNAc). Pentapeptide side chain is added to UDP-Mur NAc by successive addition of L-alanine, D-glutamate, meso-diaminopimelic acid and D-alanyl-alanine dipeptide catalysed by ATP-dependent ligases—MurC, MurD, MurE and MurF, respectively. Synthesis of D-amino acids is catalysed by specific racemases.

For transport of these precursors across the inner membrane, a C₅₅ polyisoprenoid carrier is attached to form lipid I, the undecaprenyl pyrophosphatyl-Mur NAc pentapeptide. Then, GlcNAc (from UDP-Glc NAc) is added to lipid I by MurG forming the final murein precursor lipid II. The enzyme flippase catalyses its transport across the inner membrane (Dotson et al. 1998; Van Heijenoort 2001).

Murein synthesis catalyses the enlargement of the sacculus by incorporating the lipid II and release of pyrophosphate. These proteins are anchored to the cytoplasmic membrane close to the N-terminus and are present as 120–220 copies per cell. They have a short N-terminal region in the cytoplasm, while the catalytic domains for transglycosylation (TG) and transpeptidation (TP) are located in the periplasm. The former are also referred to as glycotransferases (GTases) and catalyse polymerization of glycan chains. The TPases catalyse the cross-linking of peptides. These are also called penicillin-binding proteins (PBPs) because of their ability to covalently bind to penicillin. Three types of murein synthases are bifunctional GTases–TPases (Class A PBPs), monofunctional TPases (Class-B PBPs) and monofunctional GTases. *E. coli* has three bifunctional synthases (PBP1A, PBP1B and PBP1C), a GTase (MgtA) and two TPases. LD-TPases are covalently attached to OM lipoprotein, Lpp (Table 2.8). For proper growth and elongation of sacculi, the cell requires hydrolases to cleave glycoside and amide bonds. In *E. coli* autolysin catalyses this reaction and at least 13 different hydrolases (autolysin) are present in the periplasm (Sauvage et al. 2008; Vollmer et al. 2008; Vollmer and Bertsche 2008; Typas et al. 2012).

Actin-like protein, MreB, is used for elongation of rod-shaped gram-negative cells. MreB forms filaments and interacts with conserved inner membrane proteins MreC, MreD and RadZ as well as lipid II synthesis enzymes MraY and MurG. A tubulin-like protein, FtsZ, regulates cell division. Two outer membrane lipoproteins LpoA and LpoB are essential for the function of PBP1A and PBP1B, the major bifunctional peptidoglycan synthases. Thus, the PG synthetic machinery on one hand receives the regulatory input from the outer membrane through LpoA and LpoB, while on the other cytoskeletal elements play an important role in PG synthesis. The LpoA and LpoB specifically bind PBP and stimulate transpeptidase

Table 2.8 Peptidoglycan synthases involved in cytoplasmic PG synthesis in *E. coli* (Typas et al. 2012)

Enzyme type	Protein	Gene	Function
1. Transferase and dehydrogenase	Mur A Mur B	<i>murA</i> <i>murB</i>	Synthesis of UDP-Mur NAc from UDP-GlcNAc
2. Amino acid ligases	Mur C, Mur D, Mur E, Mur F, Ddl		Cytoplasmic synthesis of UDP-Mur NAc-pentapeptide
3. Racemases	Alr, DadX, Mur I		Synthesis of D-ala, D-gmt from L-ala, L-gmt, respectively
4. GTases	Mra Y, Mur G		Lipid II synthesis from UDP-Mur NAc pentapeptide on the inner leaflet of cytoplasmic membrane
5. GTases- and DD-Tpases (Class A PBPs)	PBP1A	PonA (<i>murA</i>)	Bifunctional transglycosylase, transpeptidase, Anchored in IM, involved in cell elongation interacts with LPOA
	PBP1B	PonB (<i>murB</i>)	α , β , γ differ with respect to the part in the cytoplasm
	α (94,292 Da)		Major peptidoglycan synthase involved in cell division
	β (91,593 Da)		Dimerizes and interacts with PBP 3, FtsN, MipA, LpoB
	γ (88,889 Da)		Anchored in IM
6. DD-Tpases (class 5B PBPs)	PBP1C (86,067 Da)	<i>pbpC</i>	Specific function not clear, cannot support growth in the absence of PBP1A an PBP1B; anchored in IM
	PBP2 (70,857 Da)	<i>pbpA</i> (<i>mrdA</i>)	Cell elongation, lateral wall and septation site Dependent on MreB filament; anchored in IM
	PBP 3 (63,877 Da)	<i>ftsI</i>	Essential for cell division, part of divisome, anchored in IM, interacts with PBP1B, MtgA, FtsQLB, FtsW and FtsN
7. GTase	MtgA (27,342 Da)	<i>mtgA</i>	Localization to the division site, interacts with PBP3, FtsW and FtsN, anchored in IM
8. Regulation and activators of peptidoglycan synthase	LpoA; LpoB		Regulate PBP 1A (LpoA) and PBP1B (LpoB) Tase activity, outer membrane lipoprotein
9. Regulation of peptidoglycan structure DDC Pases (Class C PBP's)	PBP5, PBP4B, PBP6, PBP6B		Play role in peptidoglycan synthesis by removal of excess pentapeptide donors in newly made peptidoglycan

Alr Alanine racemase, biosynthetic, *CPase* carboxypeptidase, *Dad X* alanine racemase, catabolic, *EPase* endopeptidase, *Ddl* D-ala-D-ala ligase, *Glc NAc* N-acetyl glucosamine, *GTase* glycotransferase, *Mur A* WDP-GlcNAc enolpyruvyl transferase, *Mur B* UDP-Mur BAc dehydrogenase, *Mur C* UDP-Mur NAc-L-ala ligase, *Mur D* UDP-Mur NAc-L-ala-D-glu ligase, *Mur E* UDP-Mur NAc-L-ala-D-glut-meso-diaminopimelic acid ligase, *Mur F* UDP-Mur NAc-tripeptide-D-alanyl-D-ala ligase, *Mur G* UDP-Glc NAc-undecaprenyl-pyrophosphoryl-Mur Ac-pentapeptide transferase, *Mur I* glut racemase, *Mur NAc* N-acetyl muramic acid, *PBP* penicillin-binding protein, *TPase* transpeptidase, *Ivy* Inhibitor of vertebrate lysozyme, *LT* lytic transglycosylase, *Mlt* membrane-bound murein transglycosylase, *MraY* UDP-Mur NAc pentapeptide phosphotransferase

Table 2.9 Hydrolases involved in peptidoglycan hydrolysis (autolysis) and their role in cell wall synthesis (Vollmer and Bertsche 2008)

Enzyme reaction	Protein and molecular wt.	Gene	Function
1. Lytic transglycosylase (LT)	Slt 70 (73,357x)	<i>slt Y</i>	Lipoproteins, anchored in outer membrane, function as major autolysis Breakdown to allow expansion of sacculus during growth Septum cleavage (Slt 70, Mlt A, Mlt B, Mlt C, Mlt D)
	Mlt A (40,411 Da)	<i>mlt A</i>	
	Mlt B (40,256 Da)	<i>mlt B</i>	
	Mlt C (40,113 Da)	<i>mlt C</i>	
	Mlt D (49,417 Da)	<i>mlt D</i>	
	Emt A (26,575 Da)	<i>emt A (mlt E)</i>	
	Mlt F (58,302 Da)	<i>mlt F</i>	
2. Amidases			
<i>N</i> -Acetylmuramoyl-L-alanine amidase (ami)	Ami A (31,412 Da)	<i>ami A</i>	ami and ami B, ami C are periplasmic septum cleavage or cell division
	Ami B (47,985 Da)	<i>ami B</i>	
	Ami C (45,634 Da)	<i>ami C</i>	
1,6-Anhydro- <i>N</i> -acetyl-muramyl-L-alanine amidase (1,6 ami)	Amp D (20,536 Da)	<i>amp D</i>	Cytoplasmic
3. Peptidases			
LD-endorpeptidases (LD-EP)	PBP4 (51,798 Da)	<i>dac B</i>	Periplasm, membrane proteolytic cleavage to PBP8
DD-carboxypeptidase (DD-CP)	PBP7 (34,245 Da)	<i>pbp G</i>	Biofilm formation
LD-carboxypeptidase (LD-CP)	MEPA (30,137 Da)	<i>mep A</i>	Periplasmic
	PBP5 (44,444 Da)	<i>dac A</i>	Inner membrane
	PBP6 (43,609 Da)	<i>dac C</i>	Cell shape maintenance Regulatory role
	PBP6 B (43,346 Da)	<i>dac D</i>	Cytoplasmic Cleavage turn over
	Ldc A (33,567 Da)	<i>ldc A (vegO)</i>	

activity for the attachment of new peptidoglycan to the sacculus and act as PBP cofactors in *E. coli* (Paradis-Bleau et al. 2010; Typas et al. 2010) (Table 2.9). PBP_{IB}–LPOB complex plays a role in cell division, while PBP_{IA}–LPOA is involved in cell elongation. Irrespective of their localization, they show inherent flexibility that they can undertake each other's function (Tanaka et al. 2007).

2.5 Inner Membrane (IM): Structure and Function

Inner membrane or cytoplasmic membrane is the inner most bilayer of the cell envelope that surrounds the cytoplasm and limits the individual cell as a separate entity. Cytoplasmic membrane occurs universally and is present in all types of living cells, the eubacteria, archaebacteria or eukarya. Inner membrane, as referred to in gram-negative bacteria, is a phospholipid bilayer, the inner leaflet which faces the cytoplasm and outer leaflet which faces the periplasm which also holds the cell wall. The inner membrane in *Pseudomonas* comprises of two electron-dense layers separated by an electron-transparent layer. Cells treated with lysozyme, which hydrolyses the peptidoglycan, lose their shape and get lysed unless maintained in isotonic solution such as 20 % sucrose. Under such conditions, they appear as spheres referred to as spheroplasts. Dissolution of outer layers of marine pseudomonads by washing under controlled conditions of ionic strength and pH has enhanced the isolation of cytoplasmic membrane free from contamination from other layers (Martin and MacLeod 1971). As compared to gram-positive bacteria, preparation of protoplasts in gram-negative bacteria is more tedious because of the presence of outer membrane comprising of phospholipid, lipopolysaccharide and lipoproteins. These have to be removed from the membranes by phenol extraction or by the use of proteinases and other agents which may also attack the cytoplasmic membrane. Cytoplasmic membranes are estimated to account for about 12 % dry weight of the cell in marine *Pseudomonas*. Purified membranes from stable protoplasts of marine *Pseudomonas* and treated with RNase and DNase showed that their chemical composition is similar to that of gram-positive bacteria.

The major lipid component of the cytoplasmic membrane is the phospholipid (which accounts for 80 % of the total lipid) and are similar to the lipids isolated from intact cells which also includes the phospholipids of the inner leaflet of the outer membrane (OM). The phospholipids of *P. aeruginosa* membrane preparations were similar to that of the marine pseudomonads. About 90 % of these were phospholipids and phosphatidylethanolamine was the major component with small amounts of phosphatidyl glycerol, diphosphatidyl glycerol and phosphatidyl glycerol phosphate.

The hydrophilic polar ends of the inner leaflet face the cytoplasm and that of the outer leaflet face the periplasm. The hydrophobic tails of the fatty acids extend to the interior of the bilayer structure. Unlike the outer membrane, the two leaflets of inner membrane are homologous. During the growth of *P. aeruginosa* on different media, some variations do occur in phospholipid composition of *P. aeruginosa*.

Invagination in cytoplasmic membrane forming mesosomes in *P. aeruginosa* has been reported (Hoffman et al. 1973). Similarity in *P. aeruginosa* membrane and membranes isolated from gram-positive bacteria has been observed with respect to structure, composition and function. This indicates general similarity in cytoplasmic membrane in all bacteria.

Pseudomonas membranes contain relatively high amount of protein which may reflect their metabolic diversity; even *P. aeruginosa* has been reported to contain 60 % protein in membrane. There may be 200 different kinds of proteins in the

phospholipid bilayer. The membrane proteins may be structural proteins, while others may be enzymes located in the membrane fractions. Besides membrane being the permeability barrier, it is the site for oxidative phosphorylation as it provides surface for oxidative reaction as well as site for chromosome attachment and replication. The membranes are involved in septum formation in cell division, biosynthesis and polymerization of cell wall and other cell envelope components, protein synthesis and DNA replication. In addition there are certain specific enzyme functions that are located on membranes of *Pseudomonas*. Certain enzymes are located on the outer surface of the cytoplasmic membrane, e.g. peptidoglycan-synthesizing enzymes. Some of the proteins are integral part and are built into the continuous phospholipid bilayer, while others may be associated by hydrophobic interactions with inner or outer aspects of the membrane. The passage of lipophilic molecules through the IM is fast because of the lipophilic bilayer; however, the passage of hydrophobic molecules requires specific proteins for their transport across the membrane (Neidhardt et al. 1990).

Proteins residing in the cell envelope (IM, periplasm and outer membrane) are involved in important cell functions, viz. cell wall assembly, synthesis and modelling of PG, nutrient uptake, energy production, adherence, motility, environmental sensing, biofilm formation, virulence and antibiotic resistance. Outer membrane proteins in gram-negative bacteria are also associated with antibiotic resistance (Casabona et al. 2013).

Genomic analysis of *P. aeruginosa* PAO1 indicate that ~38 % of genome (2123 ORF's) encode for proteins that are exported out of the cytosol into the cell envelope or extracellular environment. With the aim to map these proteins, *P. aeruginosa* was subjected to proteomic analysis of the membrane proteins. A total of 786 proteins were identified. Of these 333 (42 %) had a minimum of one transmembrane domain, and 195 (25 %) were classified as hydrophobic. Key integral proteins of IM and OM that are involved in antibiotic resistance by efflux pump have been identified (Blander et al. 2004; De et al. 2009). The inner membrane efflux system encoded by *mexAB-oprM* operon exports a range of antibiotics such as tetracycline, chloramphenicol, quinolones, novobiocin, macrolides, trimethoprim and β -lactams (Srikumar et al. 1997). The tripartite efflux pumps consist of IM component (MexB, MexD or MexF) which function as a resistance-nodulation division (RND) in combination with OM channel-forming components OprM, OprJ or OprN and a membrane fusion protein that forms the link between the membrane and efflux components (MexA, MexC or MexE) (Zavascki et al. 2010; Casabona et al. 2013).

Protein mapping of the inner membrane indicates that IM harbours proteins for cell biogenesis and functions such as multicomponent transporters, protein sensors, proteins that are part of the signaling and regulatory systems and protein export machinery involved in virulence (Nouwens et al. 2000, 2003). A core list of 991 nonredundant proteins was analysed in terms of transmembrane domains, signal peptide and lipobox sequence prediction. Forty-seven per cent of the identified proteins harbour at least one predicted transmembrane domain, 20 % of the proteins exhibit signal peptide allowing their export across IM, and 62 are

lipoproteins according to the recent list of *P. aeruginosa* proteins harbouring lipobox sequences. The proteins from IM repertoire were categorized with respect to cellular localization demonstrated in *P. aeruginosa* or for paralogs or similar proteins in other organisms. Majority of the proteins were associated with IM and nearly half were of unknown localization. For proteins with already described localization, three main groups were IM (10.4 % of the identified proteins), OM vesicles (OMVs 15.7 %) and multiple localizations (14.2 %), i.e. either IM or OMV. Functional categorization of the 52.5 % of the identified proteins was involved in secretion and transport (19 %) as well as biosynthesis and metabolism (33 %). Operons for proteins of type II (Xcp) and type IV secretion (Hcp secretion) were detected readily; others included type 4 pili (*pil*), cytochrome oxidase (*cco*), NAD dehydrogenase (*nuo*) complexes, proteins involved in chemotaxis (*pct*), transporters such as Bra and Fts involved in cell division and cell wall biogenesis (Casabona et al. 2013). Earlier Jagannadham and Saranya (2011) analysed 1479 membrane proteins including 179 of OM and compared their localization at different sites within the cell envelope (Table 2.10).

Proteomic changes in response to chromium (VI) toxicity showed overexpression of stress proteins, proteins involved in protein biosynthesis, proteins for energy production, proteins involved in free radical detoxification and OM proteins MucD (role in exopolysaccharide synthesis), while isocitrate dehydrogenase and 30S protein S1 were downregulated (Kilic et al. 2010). Similarly, phenol toxicity of *P. putida* KT2440 resulted in upregulation of oxidative stress response (AhpC, SodB, Tpx and Dsb), general stress response (UspA, HtpG, GrpE and Tig), energy metabolism (AcbB, AtpH, Fpr, AceA, NuoE and MmsA-1), fatty acid biosynthesis (FabB, AccC1 and FabBx1), inhibition of cell division (MinD), cell envelope biosynthesis (LpxC, VacJ and MurA), transport of small molecules (TolC, BraC, AotJ, FbpA, OprQ) and transcription regulation (OmpR and Fur). The genes involved in nucleotide synthesis (*purM*, *purI*, *pytH* and *did*) and cell motility (*fliC*) were downregulated (Santos et al. 2004).

Baysee et al. (2005) reported that adaptation of *P. aeruginosa* is a consequence of major physiological change involving cell envelope, e.g. quorum sensing can be activated by nutritional stress, independently of cell density. A link between membrane properties and stress signaling has been established. Inactivation of *lptA* gene altered the fatty acid profile of phospholipid and membrane properties resulting in decreased membrane fluidity; *lptA* gene encodes an enzyme that catalyses the second step in phospholipid biosynthesis pathway.

2.6 Biogenesis of Cell Envelope

Cell envelope of bacteria serves as the interface with the environment and has an important role in maintenance of integrity of the cell and protection from the external stresses. Structure of cell envelope in gram-negative bacteria is typically complex and multifunctional and varies throughout the bacterial domain. In pathogenic organisms cell envelope is the first to come in contact with the host and OM also provides for intrinsic resistance to antibiotics in Proteobacteria (Delcour

Table 2.10 Comparison of subcellular localization of proteins from different *Pseudomonas* sp. (Jagannadham and Saranya 2011)

S. No.	Localization	<i>Pseudomonas aeruginosa</i> PA7	<i>Pseudomonas aeruginosa</i> PAO1	<i>Pseudomonas entomophila</i> L48	<i>Pseudomonas fluorescens</i> Pf-5	<i>Pseudomonas fluorescens</i> Pf0-1
1	Cytoplasmic	2698	2593	2288	2637	2508
2	Cytoplasmic membrane	1336	1276	1179	1487	1356
3	Extracellular	55	69	51	62	73
4	Outer membrane	177	172	140	171	130
5	Periplasmic	167	170	158	214	184
6	Unknown	1698	1140	1180	1421	1332
7	Unknown (multiple localization sites)	155	146	138	146	139
8	Total	6286	5566	5134	6138	5722

2009; Tokuda 2009). Thus, the understanding of biogenesis of cell envelope is important from the point of view of interaction with both the environment and pathogenicity. All the components of the cell envelope are synthesized either in the cytoplasm or inner surface of the IM and are flipped across the IM for polymerization in the periplasm. All these precursor proteins have a signal sequence at the amino terminus, called lipobox (Driessen and Nouwen 2008). The lipobox consists of four amino acids, namely, Leu-Ala/Ser-Gly/Ala-Cys, with Cys as the first amino acid of the mature lipoprotein (Hayashi and Wu 1990; Remans et al. 2010). The translocation from cytoplasm is catalysed by a heterotrimer, IM-protein complex, and five different systems for envelope biogenesis have been identified in *E coli* and other Proteobacteria. These include (1) the Sec system that transports proteins across the inner membrane or inserts them into it, (2) the Lol system for lipoprotein transport to the outer membrane, (3) the Bam system for outer membrane beta-barrel protein assembly, (4) the Lpt system for lipopolysaccharide (LPS) transport to and assembly in the outer membrane and (5) the penicillin-binding proteins (PBPs) and associated factors that construct the PG layer (Daniel and Errington 2003; Silhavy et al. 2010; Zimmer et al. 2008).

The Sec translocon comprises of SecYEG and SecD, SecE and YajC which facilitate the release of secreted proteins into the periplasm. In the periplasm the translocated proteins are protected against misfolding and aggregation. Periplasmic chaperones function to protect OMPs during their transit through periplasm. Three such proteins with general chaperone activity have been identified, viz. SurA that also functions as peptidyl-proline isomerase (Bitto and McKay 2003), SkP (Walton et al. 2009) and DegP (Shen et al. 2009). They function in parallel pathways. SurA functions in one pathway and DegP/SkP functions in the other. SurA pathway is the preferred pathway for major OMPs (Sklar et al. 2007). As yet, no OMP have been identified to prefer DegP/Skl pathway.

Periplasmic chaperones deliver OMPs to the Bam complex in the OM. The Bam complex is composed of a large β -barrel protein, BamA (aka YaeT or Omp85), and four lipoproteins, BamBCDE (aka YfgL, NlpB, YfiO and SmpA, respectively) (Wu et al. 2005; Sklar et al. 2007). The β -barrel domain BamA has a large amino-terminal periplasmic domain composed of five POTRA (polypeptide transport associated). Structural analysis of a large fraction of the BamA periplasmic domain shows each of the four visible POTRA domains has a nearly identical fold, although they do not have much of the amino acid sequence identity (Kim et al. 2007). Bam D is the only essential lipoprotein in Bam complex and is highly conserved in gram-negative bacteria. Other three lipoproteins are non-essential. The lipoproteins are made with an amino-terminal signal sequence and are translocated by the Sec machinery. The signal sequence is later removed by a different signal peptidase. Once the signal sequence is removed, an additional fatty acyl chain is added to the cysteine amino group. The lipid moieties hold the lipoprotein to the outer leaflet of the IM and some lipoproteins remain in the IM. Lol system is responsible for the transfer of lipoproteins to the outer membrane. The Lol system comprises of an ABC transporter (LolCDE) in the IM that utilizes ATP hydrolysis to extract the molecule from IM and pass it on to LolA, a

Table 2.11 Enzymes and proteins involved in cell morphogenesis and cell division in *E. coli*

Enzyme activity	Protein	Function
Cytoskeletal structure, ATPase, GTPase	MreB	• Cell elongation • Actin structural homologue • Forms a cytoplasmic, membrane-attached helix or patches
MreB-associated proteins	MreC, MreD, Rod Z, Rod A, PBP 2	MreB-associated and IM-associated proteins (MreC, MreD, Rod Z) lipid II flippase (Rod A)
Cell division		
Cytoskeletal structure, GTPase	Fts Z	Tubulin structural homologue Forms a dynamic cytoplasmic ring in mid cell
Early association with the Z ring	Fts A, Zip A, Zap A, Zap B, Zap C, Fts X, Fts K	Stabilization and membrane attachment of Fts Z polymers (Fts A, Zip A, Zap A, Zap B, Zap C); requirement of proteins and DNA transport (Fts K)
Late association with the Z ring	FtsQ, FtsL, FtsB, FtsW, FtsN, PBP3, Dam X, Ded D, Rlp A	• Interaction with peptidoglycan synthases PBP 3 (FtsQLB, FtsW, FtsN) and PBP 1B (PBP 3 and FtsN); lipid II flippase (FtsW) • Peptidoglycan binding (FtsN, Dam X, Ded D and Rlp A)
Outer membrane invagination	TolQ, TolR, TolA, TolB, Pal	• Forms an envelope-spanning complex for outer membrane invagination during septation; peptidoglycan binding (Pal)

periplasmic carrier, which delivers the molecule to the OM assembly site, the lipoprotein LolB (Takaguchi et al. 2005).

A separate protein translocation system, Tat, in the IM translocates folded proteins (Sargent et al. 2006). The tat system is used for translocation of a small group of proteins that have prosthetic groups which must have been added in the cytoplasm. This simple system comprising of TatABC is used extensively by thermophiles. TatB and TatC target the proteins and TatA translocates it across the cytoplasmic membrane. The proteins destined for IM are also handled by the Sec machinery (Table 2.11).

The proteins are targeted for cotranslational translocation by signal recognition particle (SRP) and the SRP receptor FtsY. In bacteria the SRP contains only one protein, Ffh (fifty-four homologues) and an RNA, Ffs (four point five S RNA). The transmembrane α -helices are characteristic of biological membranes. The first transmembrane segment functions as a signal sequence to initiate translocation of sequence that follow it. These sequences serve as basis for SRP recognition and remain attached. The second transmembrane helix functions to stop translocation reaction, and this helix exits the SecYEG translocator laterally where it remains in the IM (Driessen and Nouwen 2008). The third transmembrane helix again functions as an uncleaved signal sequence. The alternate start and stop translocation signals fix the IM proteins in the membrane in a stepwise fashion. Small IM

proteins, especially those with small periplasmic domains, can be inserted into the membrane by a second translocase called YidC, which plays an important role in the assembly of energy-transducing membrane proteins.

The lipopolysaccharide which forms the outer leaflet of OM is synthesized on the inner leaflet of the IM and is flipped to the outer leaflet of IM by ABC transporter MsbA. The O-antigen is synthesized on a polyisoprenoid carrier, which then flips it to the outer leaflet. The O-antigen is ligated to the LPS core in the outer leaflet of the IM and the reaction is catalysed by WaaL (Raetz and Whitfield 2002). On the basis of biochemical and genetic studies combined with bioinformatics, seven proteins required for transport of LPS from the outer leaflet of the OM to the cell surface have been identified. These are termed Lpt (lipopolysaccharide transport) proteins and are designated as LptA (aka YhbN), LptB (aka YhbG), LptC (aka YrbK), LptD (aka Imp or OstA), LptE (RlpB), LptF (aka YjgP) and LptG (aka YjgQ). The large β -barrel protein LptD and lipoprotein LptE form a complex in OM. LptA is made with cleavable signal sequence and remains in the periplasm. LptF and LptG are IM proteins that interact with cytoplasmic protein LptB, a predicted ATPase, to form ABC transporter that together with LptC extracts LPS from the IM and passes it to the periplasmic protein LptA for delivery to the assembly site LptD and LptE in the OM. An alternate model is that all the seven proteins form a trans-envelope machine that transports LPS in a manner analogous to efflux pump. All the seven proteins are important and if anyone is lacking, LPS accumulates in the outer leaflet of IM (Sperandeo et al. 2008; Ruiz et al. 2008). The phospholipids are also synthesized in the inner leaflet of IM. MsbA can flip these molecules to the outer leaflet of the IM and then probably diffuse to the OM.

2.7 Conclusion

The cell envelope of *Pseudomonas* although similar to other gram-negative bacteria has an important role to play in the environment, pathogenicity and molecular diversity. The cell responds to the outside environmental stresses by up- or down-regulating certain proteins. Nearly 1/3 of the cell genome is involved in cell envelope biosynthesis and functions. Thus, it is not only a structural component but has a vast range of physiological functions such as transport of nutrients, intrinsic antibiotic resistance, biosynthesis and assembly of macromolecules, regulation and adhesion to the host cell and pathogenesis.

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Partap Bir Singh, Harvinder Singh Saini, and Rachhpal S. Kahlon

Contents

3.1	Introduction	82
3.2	Central Metabolic Pathways (CMP)	83
3.2.1	Carbohydrates Transport Through Membrane	84
3.2.2	Carbohydrate Catabolism	86
3.3	Peripheral Metabolic Pathways	94
3.3.1	Amino Acid Metabolism	94
3.3.2	Metabolism of Alkanes	109
3.3.3	Degradation of Aromatic Hydrocarbons	113
3.4	Conclusion	119
	References	120

Abstract

The members of the genus *Pseudomonas* have varying genome sizes ranging from 3.7 to 7.1 Mb which may contain up to 6396 predicted genes. This versatility in genome explains their ability to converge vast array of organic compounds, ranging from simple sugars to complex aromatic hydrocarbons, into the central intermediary metabolism using species-specific peripheral metabolic pathways. Further, different species of this genus may also have the potential to produce various biomolecules, viz., phytotoxic/antimicrobial compounds, siderophores, biosurfactants, and bioinsecticides, to compete with other populations in the ecosystem. Such a metabolic diversity helps these organisms to adapt and survive in wide range of ecological niches. The understanding of this intricate network of pathways provides valuable information regarding

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species known to cause deadly diseases like cystic fibrosis/nosocomial infections or beneficial in bioremediation of polluted sites and plant growth-promoting activities. Such a data could be exploited to improve the quality of human life. This chapter provides an overview regarding diversity of the metabolic pathways which are instrumental in making members of genus *Pseudomonas* one of the most successful and abundant organisms on the earth.

3.1 Introduction

The genus *Pseudomonas* a gram-negative aerobic *Gammaproteobacteria* of family *Pseudomonadaceae* has more than 190 species, and due to their widespread occurrence, these bacteria were isolated/identified early in the history of microbiology. The metabolic diversity of these bacteria allow them to colonize broad range of niches, e.g., *Pseudomonas aeruginosa* is an opportunistic human pathogen, *P. syringae* a plant pathogen, *P. putida* an efficient soil inhabitant, *P. fluorescens* a plant growth-promoting bacteria associated with plants, etc. Members of this genus can adapt in major natural environments such as soil, freshwater, and marine habitats and may associate with plants and animals, thus presenting their physiological and genetic adaptability. Pseudomonads using their peripheral metabolic pathways (described in Sect. 3.3) can degrade/utilize a broad range of toxic/nontoxic compounds. Palleroni and Doudoroff (1956) reported the growth characteristics of 267 strains of *Pseudomonas* on 146 different organic compounds indicating remarkable metabolic diversity in genus *Pseudomonas*. These bacteria are known for their efficiency to catabolize fructose, mannose, galactose, glycerol, petroleum hydrocarbons, and complex xenobiotic compounds such as benzoates, benzenes, cyclohexanes, phenols, toluene, xylenes, naphthalene, polyaromatic hydrocarbons (PAH), various pesticides, etc. which may be attributed to their peripheral metabolic pathways. Diversity in their physiological traits is also reflected at the genetic level as genome sizes within the genus vary from 3.7 Mb for *Pseudomonas stutzeri* to 7.1 Mb for *Pseudomonas aeruginosa* with 4237 to 6396 predicted genes (Ginard et al. 1997). Most genes are either specific to a species or shared by a subset of the species and this flexibility of *Pseudomonas* genome helps in adaptation of individual strain to a specific set of conditions. This diversity emerged due to variations in ecological (substrate availability, complex environment, competition etc.) and genetic factors of the pseudomonads. These variations attributed to genetic drift in pseudomonads are caused by mutation, horizontal gene transfer, and recombination.

Pseudomonads have a flexible metabolism mostly respiratory/aerobic and never fermentative; however, these bacteria may grow in the absence of O_2 by using nitrate as terminal electron acceptor, e.g., *P. denitrificans*, *P. aeruginosa*, *P. stutzeri*, etc. The process is called denitrification which converts NO_3 to N_2 by a series of steps catalyzed by reductases. The virulence of opportunistic pathogen

P. aeruginosa is also related to denitrification process. *P. aeruginosa* forms biofilm-like microcolonies in cystic fibrosis lung where environment is micro-aerobic to anaerobic and availability of nitrate favors the formation of more rich biofilms by these bacteria (Lam et al. 1980). On the other hand, complete denitrification by pseudomonads in the environment is an advantageous process as incomplete denitrification produces nitric oxide and nitrous oxide which are green house gases responsible for acid rain. Further, denitrification by these bacteria in biological wastewater treatment may help to reduce eutrophication.

The metabolically diverse pseudomonads have ability to produce wide varieties of secondary metabolites such as siderophores (pyochelin, pseudomonine, paerucumarin, etc.), biosurfactants (glycolipids, phospholipids, lipopeptides, etc.), phytotoxic compounds (tabotoxins, phaseolotoxin, safracin, etc.), antimicrobial compounds (pyrrolnitrin, polyketides, mupirocin, etc.), and certain plant growth-promoting factors. These secondary metabolites help *Pseudomonas* in nutrient acquiring, pathogenicity, competition, and defense against predation in natural environment.

Thus, understanding biochemistry and metabolic pathways of pseudomonads may be beneficial in controlling pathogenicity caused by these bacteria, detoxifying the surrounding by exploitation of their potent pollutant-degrading skills for bioremediation of polluted sites and production of useful secondary metabolites (siderophores, antibiotics, plant growth promoters, etc.) at industrial scale. Moreover, their metabolic pathways can be modified by varying their well-understood genetic pattern to hyper-produce a desired compound or synthesize useful synthetic compound of economic importance.

3.2 Central Metabolic Pathways (CMP)

The central metabolic pathway in genus *Pseudomonas* involves catabolism of carbohydrates and anabolism of amino acids and nucleotides. The members of genus *Pseudomonas* have the ability to catabolize different sugars (glucose, gluconate, glycerol, glycerate, fructose, and mannitol). Thus, central metabolic pathways involve breakdown of carbohydrates and carboxylic acids which provides precursor metabolites for other catabolic and anabolic pathways. The metabolism of carbohydrates follows three major central metabolic pathways: Embden–Meyerhof–Parnas pathway (glycolysis), pentose phosphate pathway (PPP), and Entner–Doudoroff (ED) pathway. These three pathways metabolize glucose to glyceraldehydes-3-phosphate which is further oxidized to pyruvate by a single set of lower EMP reactions. The intermediates of peripheral pathways may enter any of the central metabolic pathways (EMP, ED, PPP, or citrate cycle) to derive energy and synthesize building blocks for the cells. This ability helps in successful adaptation of pseudomonads at multiple niches, particularly nutrient-poor conditions which is an advantage in outgrowing competitors belonging to different groups (Kiewitz and Tümmler 2000). The organisms belonging to diverse groups show similarity in these basic metabolic pathways and their components which is a

prominent feature of metabolism. These similarities in central metabolic pathways are due to their early appearance during evolution and were retained in different species because of their need and efficacy.

3.2.1 Carbohydrates Transport Through Membrane

In *Pseudomonas*, four substrate-selective porins were identified: OprD porin for diffusion of basic amino acids and gluconate; OprP and OprO for phosphate and polyphosphate, respectively; and OprB, a carbohydrate-selective porin which facilitates the diffusion of glucose and other wide range of carbohydrates (Wylie and Worobec 1994; Llamas et al. 2003). However, in pseudomonads, intake of solutes across the plasma membrane may also take place by active transport system which transports solute without its modification across the membrane against concentration gradient. Another prominent mechanism of transport is by “phosphoenolpyruvate group translocation” of solute which involves chemical modification of solute and induces phosphotransferase system (PTS) for transport of solutes.

In pseudomonads, the only PTS reported is for fructose which transport fructose across the plasma membrane by converting fructose into fructose-1-phosphate, and energy for this process comes from phosphoenolpyruvate (Sawyer et al. 1977). The benefit of this transformation is that, unlike fructose, fructose-1-phosphate will not leak out of the cell which provides a one-way concentration gradient of fructose inside the cell. The presence of such a PTS for fructose allows its catabolism via both Entner–Doudoroff pathway (52.0 %) and Embden–Meyerhof–Parnas pathway (34.0 %) (Chavarria et al. 2013). However, pseudomonads lack such a PTS for hexose sugars including glucose which affects its effective uptake by the cells (Romanoa et al. 1970). Pseudomonads during growth using mannitol as sole carbon source induce an active transport system and mannitol dehydrogenase converts mannitol to fructose. Moreover, radiochromatography presented activity of inducible fructokinase by cells of *P. aeruginosa* PAO on either mannitol or fructose, but no such induction was observed when glucose was used as a sole carbon source (Eagon and Phibbs 1971).

The intake of glucose through plasma membrane involves two different pathways: one is the direct oxidative pathway, and the other is nucleotide-dependent phosphorylative pathway (Fig. 3.1). In direct oxidative pathway, glucose in periplasmic space gets converted into gluconate which is further converted into 2-ketogluconate by the activities of glucose dehydrogenase (Gcd) and gluconate dehydrogenase (Gad) respectively present in plasma membrane of *Pseudomonas* (Roberts et al. 1973; Lessie et al. 1979). The resulting gluconate and 2-ketogluconate are transported across the inner membrane by gluconate permease (Gntp) and 2-ketogluconate transferase (Kgt) which are carrier-mediated transport systems coupled to proton motive force. The inducer for their transport is not glucose but gluconate. On the other hand, in phosphorylated pathway, as PEP-glucose PTS is not present in *Pseudomonas*, glucose is directly transported across the inner membrane as free sugar by plasma membrane-mediated glucose

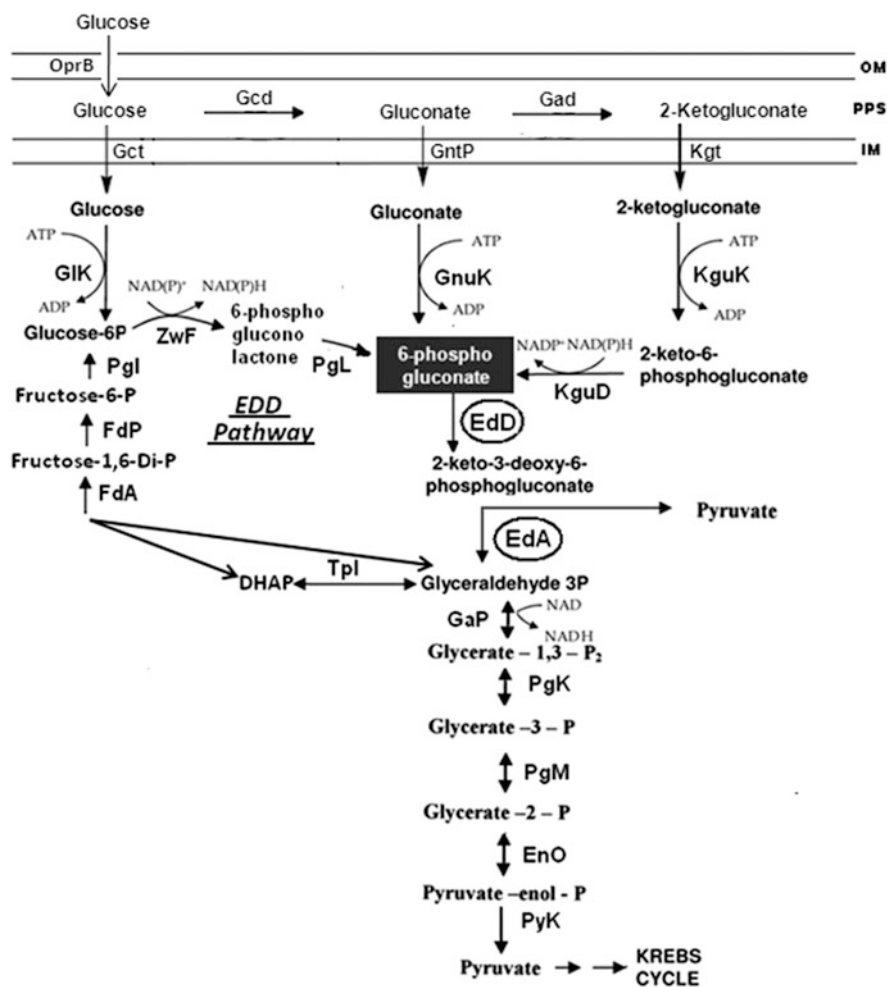


Fig. 3.1 Summary of glucose metabolism via ED pathway: The uptake and catabolism of glucose via ED pathway involve enzymatic system which is comprised of Gcd, glucose dehydrogenase; Gad, gluconate dehydrogenase; Gct, glucose transferase; GntP, gluconate permease; Kgt, 2-ketogluconate transporter; *glk*, glucokinase; *zwf-1*, glucose-6-phosphate 1-dehydrogenase; *gnuK*, gluconokinase; *kguK*, 2-ketogluconate kinase; *kguD*, 2-ketogluconate reductase; *pgl*, 6-phosphogluconate lactonase; *edd*, phosphogluconate dehydratase; *eda*, 2-keto-3-deoxy gluconate aldolase; *tpi*, triosephosphate isomerase; *fda*, fructose diphosphate aldolase; *fdp*, fructose diphosphatase; and *Pgi*, glucose-6-phosphate isomerase. The reaction further proceeds to pyruvate via lower EMP pathway involving *gap*, glyceraldehyde-3-phosphate-dehydrogenase; *pgk*, phosphoglycerate kinase; *pgm*, phosphoglycerate mutase; *eno*, enolase; and *pyk*, pyruvate kinase. OM, outer membrane; PPS, periplasmic space; IM, inner membrane

transferase (Gct) and rapidly metabolized to glucose-6-phosphate by glucokinase (Glk) at the expense of one ATP. The NADP-dependent glucose-6-phosphodehydrogenase (Zwf) then converts glucose-6-phosphate to 6-phosphogluconate which may enter ED pathway.

3.2.2 Carbohydrate Catabolism

The catabolism of carbohydrate that fuels aerobic growth of *Pseudomonas* includes three major pathways:

1. Entner–Doudoroff (ED) pathway
2. Pentose phosphate pathway (PPP)
3. Tricarboxylic acid (TCA) cycle

The reducing power generated during catabolism of carbohydrate specifically in TCA cycle is used for synthesis of ATP by employing proton motive force via oxidative phosphorylation.

Genes responsible for catabolism of glucose in pseudomonads are organized in series of clusters on the chromosome. Gene *glk* is coding for glucokinase located in an operon with *edd* gene coding for 6-phosphogluconate dehydrogenase. Similarly, *zwf* gene encoding glucose 6-phosphate dehydrogenase formed on operon with *eda* gene coding for 2-keto-3-deoxy-6-phosphogluconate. Therefore, the genes of the glucokinase pathway and Entner–Doudoroff pathway are physically linked (del Castillo et al. 2008). Doudaoua et al. (2010) reported that genes coding for periplasmic and cytoplasmic set of proteins are clustered on the host chromosome and grouped within two independent operons under the control of PtxS regulator. The two operons are inducible by glucose, gluconate, and 2-ketogluconate. However, in vitro studies showed that only 2-ketogluconate binds to the regulator. The PtxS is made of two domains, a helix–turn–helix DNA binding domain located at the N terminal and a C terminal domain that binds to the effector.

3.2.2.1 Entner–Doudoroff (ED) Pathway

The Entner–Doudoroff pathway was first discovered in 1952 by Nathan Entner and Michael Doudoroff in *Pseudomonas saccharophila*. The common occurrence of Entner–Doudoroff (ED) pathways among saccharolytic archae and absence of the conventional Embden–Meyerhof–Parnas (EMP) indicated that the ED pathway is the older route of carbohydrate catabolism. The work of Entner and Doudoroff revealed that C1 and C4 carbons of glucose were oxidized to give CO₂ in ED pathway rather than C3 and C4 carbons as in the case of EMP pathway.

The ED pathway was discovered as a series of reactions alternate to EMP that catabolize glucose, fructose, and mannitol, and it distinctively present in prokaryotes only (Entner and Doudoroff 1952; Palleroni and Doudoroff 1956). The substrates transported across the plasma membrane, i.e., glucose, fructose, mannitol, and gluconate or 2-ketogluconate, get converted into a single product

6-phosphogluconate which is an initial substrate for ED pathway (Fig. 3.1). Glucose may be transported by binding to periplasmic binding protein and phosphorylated by enzyme glucokinase (GlcK). The glucose is first oxidized to gluconate by glucose dehydrogenase (Gcd) in the periplasmic space than involves phosphorylation of gluconate mediated by gluconokinase (GlcK). Third is the ketogluconate loop in which case gluconate is converted into 2-ketogluconate on the cell surface and is transported inside the cell by specific transport mechanism involving 2-ketogluconate transporter and phosphorylated by 2-ketogluconate kinase to form 2-keto-6-phosphogluconate. Del Castillo et al. (2008) carried out enzymatic and microarray analysis that in *P. putida* three peripheral glucose pathways are induced by glucose in the medium. Metabolic flux analysis in *P. putida* KT2440 indicated that 50 % of glucose taken up by the cells is channelized through 2-ketogluconate peripheral pathway. 2-Ketogluconate is formed in the periplasm through reactions catalyzed by glucose dehydrogenase and gluconate dehydrogenase. 2-Ketogluconate is transported to cytoplasm and pathway converges at 6-phosphogluconate. The genes for the periplasmic and cytoplasmic are clustered and lie within two independent operons under the control of PtxS regulator (Doddoua et al. 2010).

There are two key enzymes of ED pathway: those convert substrate 6-phosphogluconate to pyruvate by a two-step reaction. The enzyme 6-phosphogluconate dehydratase (*Pgd* or *Edd*) transforms 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate which further act as a substrate for second key enzyme 2-keto-3-deoxy-6-phosphogluconate aldolase (*Kdga* or *Eda*) and transformed to glyceraldehyde-3-phosphate and pyruvate. Further, glyceraldehyde-3-phosphate may proceed to pyruvate via lower EMP pathway yielding NADH and ATP. The enzyme EDD and EDA were first time purified partially from *P. fluorescence* and characterized. The optimum pH for the activity of EDD was found to be 8.0, and it activates in the presence of divalent cations Fe^{2+} , Mn^{2+} , and Mg^{2+} , while EDA acts optimally between pH 7.0 and 8.5 (Kovachevich and Wood 1954). The ED pathway allows cyclic operation in genus *Pseudomonas* where glyceraldehyde-3-phosphate is recycled to 6-phosphogluconate via gluconeogenic enzymes, i.e., triosephosphate isomerase, fructose diphosphate aldolase, fructose diphosphatase, and phosphoglucoisomerase. The cyclic ED pathway helps in generation of more reducing power (NADPH). The enzyme 6-phosphogluconolactonase is an essential enzyme of the cyclic Entner–Doudoroff pathway activity which is induced in *P. aeruginosa* PAO1 by growth on mannitol and repressed by growth on succinate. The ED pathway during the process yields one ATP, one NADH, and one NADPH for each glucose molecule, whereas glycolysis has a net yield of two ATP and two NADH at the expense of one glucose molecule.

Thus, ED route is more efficient as compared to EMP pathway in generation of reducing power in terms of cofactor NADPH during conversion of gluconolactone-6-phosphate to 6-phosphate-gluconate (Conway 1992; Kim et al. 2008). The cofactor NADPH is predominately involved in synthesis of various amino acids and counteracting oxidative stress in pseudomonads (Singh et al. 2007). Aerobic

respiration involving O_2 as terminal electron acceptor can enhance the generation of NADH and synthesis of ATP (higher for EMP pathway), but this is also accompanied by the formation of reactive oxygen species (ROS), i.e., superoxides ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (Imlay 2008). The incomplete reduction of oxygen by NADH oxidase can result in generation of superoxide anion ($O_2^{\cdot-}$) which can be easily transformed to H_2O_2 or the hydroxyl radical ($HO^{\cdot}$) either spontaneously or by the activity of superoxide dismutase. However, in aerobic microorganisms, ED pathway may help in lowering the ROS production as this pathway maintained low NADH/NAD level as compared to EMP. In addition, it supports high NADPH redox to activate the antioxidant defense system followed by ROS sequestration under aerobic conditions (Aon et al. 2010).

Mannose and fructose follow the metabolic pathway analogous to glucose (Fig. 3.2). Specific hexokinase phosphorylates these to mannose-6-phosphate and fructose-6-phosphate which are subsequently isomerized to glucose-6-phosphate. Glucose-6-phosphate via 6-phosphogluconate is converted to 2-keto-3-deoxy-6-phosphogluconate which split into pyruvate and 3-phosphoglyceraldehyde. Alternatively, 6-phosphogluconate can enter pentose pathway and yield one molecule of ribulose-5-phosphate and one molecule each of CO_2 and NADPH. Enzyme 6-phosphogluconate dehydrogenase through a sequential pathway oxidized 6-phosphogluconate to 3-keto-6-phosphogluconate and then decarboxylated to form D-ribulose-5-phosphate. This pathway is important as it enables the organism to produce pentose precursors for synthesis of purines, pyrimidines, and aromatic amino acid by reversed hexose monophosphate pathway.

In *P. fluorescens* glucokinase activity was not detected in glucose- or gluconate-grown cells (Quay et al. 1972). Glucose grown cells showed the presence of membrane-bound glucose oxidase. The activity of this enzyme was very low in gluconate-grown cells. Both glucose- and gluconate-grown cells contained gluconokinase and 6-phosphogluconate dehydratase, but in mutants lacking glucose oxidase, these two enzymes could be induced by gluconate but not by glucose. This indicated that in *P. fluorescens* gluconate is the inducer of ED pathway enzymes rather than glucose. Other enzymes induced were gluconate oxidase, 6-phosphogluconate dehydrogenase, and glucose-6-phosphate-dehydrogenase.

P. saccharophila metabolizes galactose by forming 2-keto-3-deoxygalactonate before any phosphorylation takes place (Deley and Doudoroff 1957). The specific kinase phosphorylates 2-keto-3-deoxygalactonate to 2-keto-3-deoxy-6-phosphogalactonate. The next step of cleavage by aldolase is similar to 2-keto-3-deoxy-6-phosphogluconate cleavage, resulting in the formation of D-glyceraldehyde 3-phosphate and pyruvate.

3.2.2.2 Pentose Phosphate Pathway

The pentose phosphate pathway is one of the three essential pathways of central metabolism for oxidation of glucose. It is divided into its oxidative portion and non-oxidative portion. The pathway begins with D-glucose-6-phosphate and ends with the formation of D-fructose-6-phosphate and D-glyceraldehyde-3-phosphate. The ED pathway connects with oxidative part of pentose phosphate through

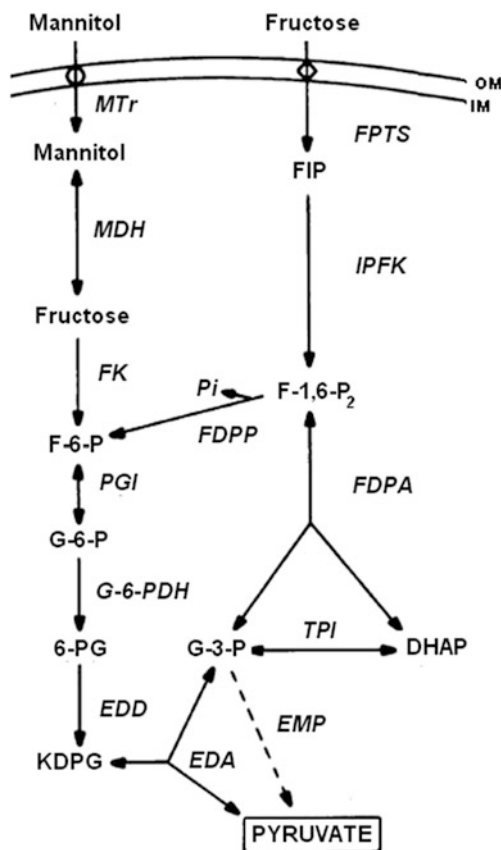


Fig. 3.2 Pathways for mannitol and fructose catabolism in *P. aeruginosa* PAO: Pathway intermediates, transport activities, and enzymes are abbreviated as follows: fructose-6-phosphate (F6P), fructose-1-phosphate (FIP), fructose-1,6-diphosphate (F-1,6-P₂), glucose-6-phosphate (G-6-P), 6-phosphogluconate (6-PG), 2-keto-3-deoxy-6-phosphogluconate (KDPG), glyceraldehyde-3-phosphate (G-3-P), dihydroxyacetone phosphate (DHAP), mannitol transport (MTr), mannitol dehydrogenase (MDH), fructokinase (FK), fructose-1-phosphotransferase system (FPTS), 1-phosphofructokinase (IPFK), phosphoglucosomerase (PGI), glucose-6-phosphate-dehydrogenase (G6PDH), 6-phosphogluconate dehydratase (EDD), KDPG aldolase (EDA), fructose diphosphate phosphatase (FDPP), fructose diphosphate aldolase (FDPA), and triosephosphate isomerase (TPI). EMP refers to the Embden–Meyerhof pathway

6-phosphogluconate which is oxidized to D-ribulose-5-phosphate. The subsequent non-oxidative portion involves conversion of D-ribulose-5-phosphate through a series of transaldolase and transketolase reactions into D-fructose 6-phosphate and D-glyceraldehyde 3-phosphate. This pathway is important for the conversion of hexoses to pentoses (Fig. 3.3).

Thus, pentose phosphate pathway supplies three of the major precursor metabolites such as D-ribose-5-phosphate, D-sedoheptulose-7-phosphate, and D-erythrose-4-phosphate which serve as precursors for synthesis of the nucleotides

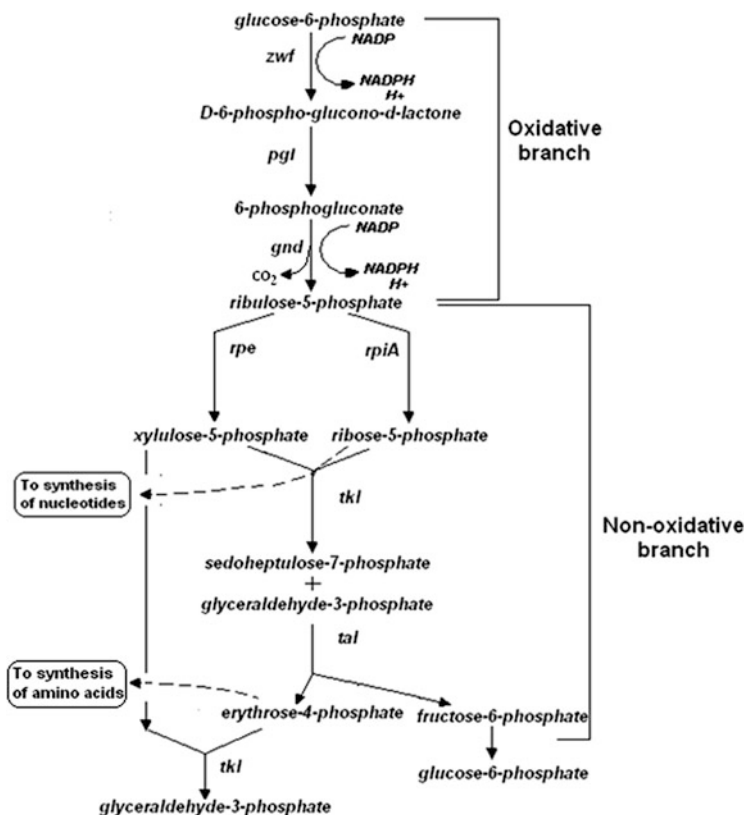


Fig. 3.3 Summary of glucose metabolism by pentose phosphate pathway: The enzymes involved during the process are glucose 6-phosphate dehydrogenase (*zwf*), 6-phosphogluconolactonase (*pgl*), 6-phosphogluconate dehydrogenase (*gnd*), ribulose 5-phosphate isomerase (*rpi*), ribulose 5-phosphate-3-epimerase (*rpe*), transketolase (*tkl*), and transaldolase (*tal*)

and amino acids. Irrespective of the carbon source utilized by the cells for their growth, some carbon has to flow via the pentose phosphate pathway to fulfill requirements of the cells for these three metabolites. On the other hand, this pathway is an important source of reducing power NADPH, needed for biosynthetic reactions where it serves as an electron donor primarily for fatty acid synthesis and siderophore synthesis and to control oxidative stress as described above in *Pseudomonas*.

3.2.2.3 Tricarboxylic Acid (TCA) Cycle

The TCA pathway is the first step in generating energy, reducing power and precursors for biosynthesis. The pathway is also known as the citric acid cycle or Szent-Gyorgyi-Krebs cycle (the Krebs cycle) named after its discoverer. The name of the TCA cycle is derived from its first step where acetyl-CoA (2C) binds to

oxaloacetate (4C) to form citrate (6C), an acid with three carboxylate groups. *P. putida* and *P. fluorescens* grow well on acetate as well as intermediates of TCA cycle. However, cells grown on acetate show lag when shifted to succinate or citrate as carbon source and succinate grown cells show lag for oxidation of citric acid. Further studies revealed that this lag was not because of lack of metabolic enzymes for the TCA cycle intermediates but due to lag in synthesis of the specific permeases required for their transport (Tiwari and Campbell 1969).

The input to the cycle is acetyl-CoA and a usual source of acetyl-CoA is pyruvate (pyruvate converted to acetyl-CoA by the pyruvate dehydrogenase complex) which is produced by the degradation of carbohydrates, fats, and proteins. Citrate synthase is an important enzyme as carbon in the form of acetyl enters TCA cycle at this stage. Apart from acetyl being formed from carbohydrate metabolism, acetate is also contributed by metabolism of fatty acids and related compounds. Thus, regulation at this step is important, and the enzyme citrate synthase is regulated by NADH and succinyl-CoA. The enzyme is also stimulated by AMP.

In the next step, citrate is converted to isocitrate. This reaction is mediated with the formation of *cis*-aconitic acid. The two steps are catalyzed by enzyme aconitate hydratase. Isocitrate is converted into oxalosuccinic acid by NADP-dependent isocitrate dehydrogenase. The same enzyme is catalyzing the conversion of oxalosuccinic acid to 2-ketoglutaric acid with release of CO₂. Isocitrate dehydrogenase is a key enzyme of the TCA cycle as isocitrate can branch off from the TCA cycle, and bypassing α -ketoglutarate via glyoxylate shunt results in formation of succinic acid and glyoxylate (Fig. 3.4). This modifies the TCA cycle where acetyl-CoA enters the cycle at two steps, but no carbon escapes in the form of CO₂. A key enzyme malate synthase condenses glyoxylate and a second molecule of acetyl-CoA to form malate (Wong and Aji 1956). Thus, it is responsible for replenishment of TCA cycle intermediates. The glyoxylate shunt allows net synthesis of one molecule of succinate from two molecules of acetyl-CoA. That allows pseudomonads to use substrates such as fatty acids, alcohols, esters, waxes, alkenes, and methylated compounds entering central carbon metabolism at the level of acetyl-CoA.

The second molecule of CO₂ arises during the conversion of α -ketoglutarate to succinate. This also involves a multienzyme complex system analogous to pyruvate dehydrogenase. The next step in the oxidation is the dehydrogenation of succinate. Succinate is oxidized to fumarate by succinate dehydrogenase; this enzyme is closely linked to electron transport chain at the level of FAD. Succinate dehydrogenase is inhibited by oxaloacetate.

Then fumarate is hydrated by fumarate hydratase to malic acid, which undergoes dehydrogenation by NAD-dependent malate dehydrogenase resulting in formation of oxaloacetate. Malate dehydrogenase is a constitutive enzyme with catabolic activity. In *P. fluorescens* and *P. ovalis*, this NAD-linked malate dehydrogenase is absent, and oxidation is linked to O₂ through a membrane-bound enzyme and requires cytochrome C.

TCA cycle has a vital role in the catabolic and anabolic reactions. The catabolic products of carbohydrates via glycerol and lactate converge at pyruvate. Fatty

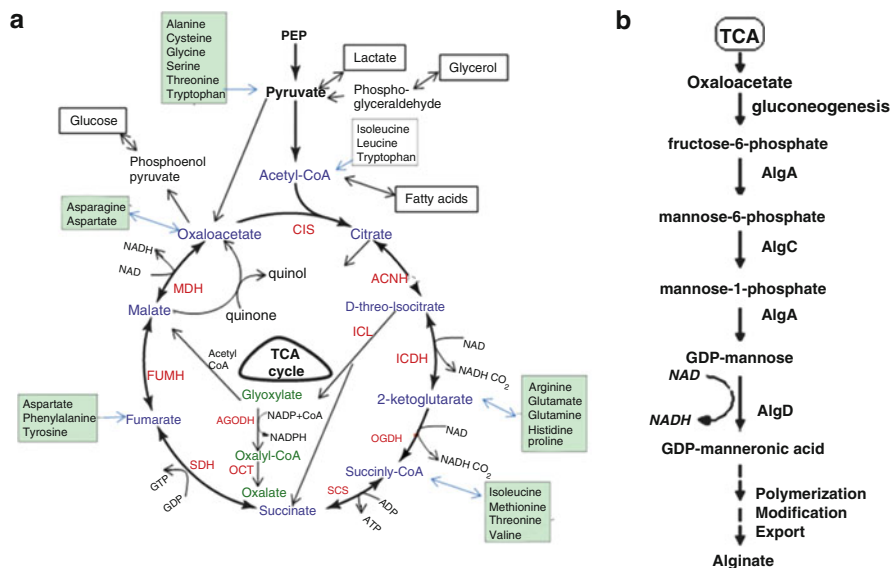


Fig. 3.4 (a) *Tricarboxylic acid cycle*: Enzymes involved are malate dehydrogenase (MDH), fumarate hydratase (FUMH), succinate dehydrogenase (SDH), succinyl-CoA synthetase (SCS), 2-oxoglutarate dehydrogenase (OGDH), isocitrate dehydrogenase (ICDH), aconitate hydratase (ACNH), citrate synthase (CIS), acylating glyoxylate dehydrogenase (AGODH), and oxalate CoA-transferase (OCT). (b) *Alginate synthesis pathway*: AlgA, phosphomannose isomerase–GDP-mannose pyrophosphorylase; AlgC, phosphomannomutase; AlgD, GDP-mannose dehydrogenase. *Dashed arrows* indicate unknown biosynthesis steps of polymerization, acetylation, export, and epimerization

acids, acetamide, and β -OH butyrate enter at acetyl-CoA. Amino acids, arginine, and histidine generate glutamate which transfers its NH_2 group to yield 2-oxoglutarate.

Each cycle of TCA converts one molecule of acetyl-CoA into two CO_2 molecules; reduces NAD^+ , NADP^+ , and quinone to NADH, NADPH, and quinol, respectively; and phosphorylates one molecule of guanosine diphosphate (GDP) to GTP. The reduced molecules of NADH/NADPH/quinol serve as electron donors for oxidative phosphorylation in aerobic respiration. The reducing power (NADH) produced in ED pathway and the TCA cycle is used to drive the synthesis of ATP by oxidative phosphorylation. The electrons move through electron transport chain and sequentially eject protons and generate the chemiosmotic gradient across the membrane called the proton motive force. ATP synthetase allows protons to move across the membrane and harvests the energy which is released in the form of ATP. The intermediates 2-oxoglutarate and succinate synthesized during the TCA pathway are important in biosynthesis of amino acids glutamate and lysine, respectively (Fig. 3.4).

The NADH accumulates in pseudomonads when terminal electron acceptor oxygen or nitrate is limiting. The bacteria maintain redox homeostasis under

these conditions by production of redox-active antibiotics called phenazines (pyocyanin, a virulence factor for eukaryotic hosts) which act as end electron acceptor. Alexa et al. (2006) observed that a mutant of *P. aeruginosa* PA14 defective in phenazine production accumulated more NADH in stationary phase than the wild type. This correlated with a decrease in oxygen availability in the stationary phase and was relieved by the addition of nitrate. Addition of pyocyanin to the mutant also decreased intracellular NADH levels, which facilitates redox balancing in the absence of other electron acceptors. However, production of pyocyanin by wild strain leads to production of reactive oxygen species such as superoxide, but *P. aeruginosa* resists this toxicity by increased activities of superoxide dismutase and catalase.

In aluminum (Al)-contaminated environment, an alternative TCA cycle appears in *P. fluorescens* where TCA cycle instead of releasing CO₂ from acetyl-CoA fixes it into oxalate by the sequential enzymatic reactions involving enzyme isocitrate lyase which converts isocitrate to glyoxylate and succinate. Glyoxylate further transformed to oxalate by series of steps involving enzymes acylating glyoxylate dehydrogenase and oxalate CoA-transferase with the release of NADPH (Fig. 3.4a). Oxalate, a dicarboxylic acid, is involved in the detoxification of Al. As under Al-stress, oxidative phosphorylation was sharply reduced due to dysfunctional Fe metabolism; in that case, the ATP budget was maintained by enhanced substrate-level phosphorylation, a process mediated by succinyl-CoA synthetase (SCS). The net effect of this metabolic adaptation by *P. fluorescens* is limited CO₂ release, decreased NADH formation, and increased production of three critical metabolites oxalate, ATP, and NADPH (Fig. 3.4a). This fine metabolic-balancing act is crucial for the survival of the microbe.

Pseudomonas aeruginosa is a known opportunistic pathogen and forms biofilm in the lungs, causing cystic fibrosis. In *P. aeruginosa*, carbon sources are oxidized to acetyl-CoA, which enters the citric acid cycle leading to production of oxaloacetate. In these pathogenic microbes, the process of gluconeogenesis converts oxaloacetate into fructose 6-phosphate, which is a precursor for alginate production (Fig. 3.4b). The production of alginate gene involved *algR2*, which is a regulatory gene coding for enzyme AlgR2, and activates the *algD* promoter under stress, i.e., starvation and low-oxygen conditions stimulate formation of mucoid colonies, thus forming biofilm-like growth in the lungs. Alginate is a viscous exopolysaccharide consisting of D-mannuronic acids and L-guluronic acids which prevent phagocytosis and protect the cells from the host's immune response.

Pseudomonas is known for its metabolic diversity to degrade aromatic compounds via catechol and protocatechuate, leading to formation of succinate and acetate which are assimilated further in the intermediary metabolism (described in Sect. 3.3.3). Thus, TCA also serves as an important link for catabolism of large number of organic xenobiotic compounds.

3.3 Peripheral Metabolic Pathways

Bacterial genomes have tendency to accumulate ecologically useful gene sequences by mutation, recombination, and lateral gene transfer and, thus, possess a diverse range of metabolic and nutrient-scavenging pathways (Lawrence 1999). The large genome size of pseudomonads reflects this concept where most strains came across a broad range of ecological niches. This allows bacterial metabolism to breakdown a wide range of natural and synthetic simple/complex compounds (carbohydrates, amino acids, fatty acids, nucleotides, and xenobiotic compounds) via peripheral reactions and incorporate intermediary metabolites into central pathways. Moreover, growth of *P. aeruginosa* on *n*-alkanes, toluene, xylene, benzoate, and other aromatic compounds reflects its metabolic potential to degrade various hydrocarbons and xenobiotics (Palleroni 1986; Hickey and Focht 1990; Marín et al. 2003). *P. multivorans* have the ability to utilize up to 108 diverse organic compounds as growth substrates which demonstrate variety of catabolic pathways available within a single *Pseudomonas* strain (Stanier et al. 1966). The detailed study of peripheral metabolic pathway of pseudomonads revealed that these microorganisms have extended the substrate range by developing “peripheral enzymes” which supports their survival in various environmental niches.

The peripheral metabolic pathways also contribute in the production of useful secondary metabolites (antibiotics, biosurfactants, siderophores, etc.) which may provide survival edge to pseudomonads in terms of competition with other microbial inhabitants and drawing nutrition from otherwise non-bioavailable sources (hydrophobic compounds).

3.3.1 Amino Acid Metabolism

There are certain distinctive features observed regarding metabolism of amino acids in pseudomonads. These may include (1) the use of repression in amino acid biosynthesis as major regulatory process and prominent dependence on end-product inhibition, (2) the absence of gene clustering of enzymes required for specific amino acid biosynthesis, and (3) the utilization of multiple pathways for amino acid metabolism.

3.3.1.1 Membrane Transport

Transport or uptake of the nutrients from the environment plays a key role in growth and survival of the organism in the presence of competition from other organisms. Thus, microorganisms use a very selective and efficient uptake system for their survival and growth. One such system is ATP-binding cassette (ABC) that has been identified both in prokaryotes and eukaryotes. ABC transporters are transmembrane proteins that utilize the energy of ATP binding and hydrolysis to carry out three main functions. As importers, membrane-spanning region of the ABC transporter provides a passage across the cell membrane and protects hydrophilic substrates from the lipids of the membrane bilayer. As “exporters” or “effluxers,” ABC

transporters function as pumps that extrude toxins and drugs out of the cell. However, the third subgroup of ABC proteins is involved in translation and DNA repair processes rather than transporter.

In bacteria, it has a key role to play in the transport of a variety of solutes like sugars, amino acids, growth factors, ions, etc. Some members of ABC transport superfamily are also involved in signal transduction, protein secretion, drug resistance, pathogenesis, etc. ABC transporters are composed of four functional modules: two transmembrane permease domains and two nucleotide-binding domains (NDB). In bacteria, the ABC importers are bound by highly specific solute-binding protein, while the ABC exporters lack the solute-binding protein (Fath and Kolter 1993; Davidson and Chen 2004). In gram-negative bacteria, the proteins which bind to solutes are dissolved in the periplasm, while in gram-positive they are membrane-anchored lipoproteins. The specificity of ABC transporters depends on the selective binding of the periplasmic receptor.

P. putida KT2440, a well-characterized metabolically versatile organism growing on a wide range of carbon and nitrogen sources, has ABC transporter which shows specificity for acidic amino acids glutamate and aspartate. The system is thus referred to as acidic amino acid transport (aat). In *P. putida* KT2440, aatJ, aatM, aatQ, and aatP are encoded by an operon-involving genes PP1068–PP1071.

The Aat system involves a periplasmic solute-binding protein aatJ, two permease domains, AatQ and Aat M, and an ATP-binding subunit AatP (Fig. 3.5). In *P. putida* KT2440, expression of *aat* depends upon σ^{54} , which is involved in transcription of genes related to nitrogen metabolism (Sonawane et al. 2006). The *aat* region is adjacent to *aau*, an operon that codes for two-compartment regulatory system AuRS. AuR–AuS system is activated by the presence of acidic amino acids and their amides. The activated two-component system AuRS functions by upregulating (1) glutaminase-encoding *ansB* for their conversion into respective amino acids; (2) Glu/Asp transporters *GltP* and the *braCDEFG* operon (PP1141–PP1137) to facilitate uptake of the Glu/Asp and branched-chain amino acids, respectively; and (3) phosphoenol pyruvate (PEP) synthase (PpSA) to stimulate gluconeogenesis from Asp and Glu (Sonawane et al. 2006).

3.3.1.2 Branched-Chain Amino Acid Metabolism

Three hydrophobic aliphatic branched-chain amino acids valine, isoleucine, and leucine can also be used as carbon and energy source by pseudomonads (Marshall and Sokatch 1972). Transport of branched-chain amino acids in *P. aeruginosa* is mediated by LIV-I and LIV-II systems. High-affinity LIV-I system is specific for transport of alanine/threonine and branched-chain amino acids. Low-affinity LIV-II is a carrier-mediated transport system coupled to Na^+ or Li^+ ions. Mutants defective in these systems are unable to uptake leucine.

The interesting feature in their catabolism in *Pseudomonas* is that the initial three catabolic steps for these three amino acids are catalyzed by a single set of three enzymes, i.e., branched-chain amino acid transaminase (BauA), branched-chain keto acid dehydrogenase (BauB), and isobutyryl-coenzyme A dehydrogenase (BauC). Amino acid transaminases and isobutyryl-CoA dehydrogenase are

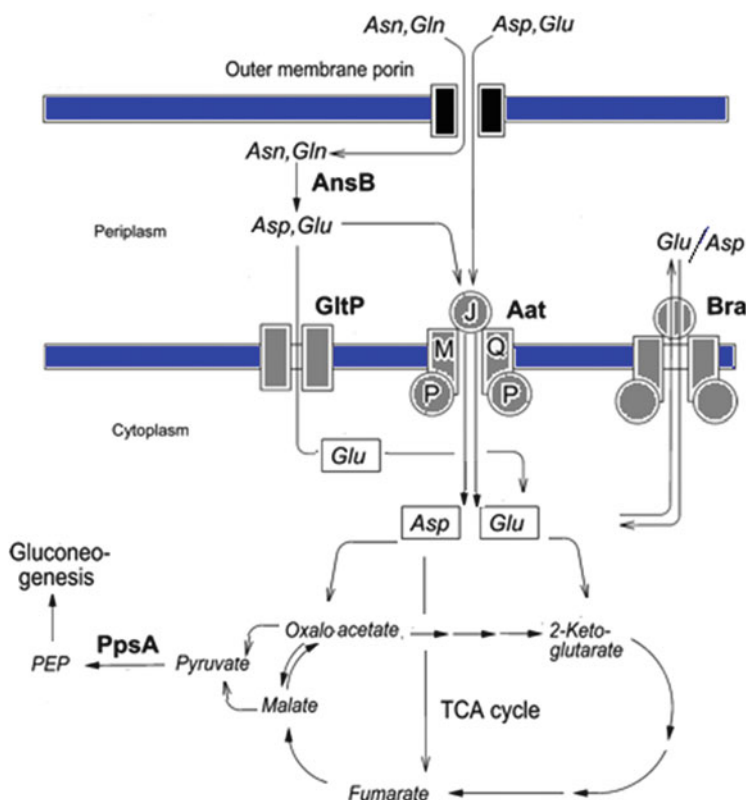


Fig. 3.5 Uptake and utilization of acidic amino acids by *P. putida* KT2440 using Aat system containing protein *aatJ*; two permease domains, *aatQ* and *aatM*; and ATP-binding subunit *aatP*; phosphoenolpyruvate (PEP) synthase (PpsA)

constitutive in nature, while other enzymes are inducible and regulated in different ways. These enzymes convert L-valine, L-isoleucine, and L-leucine into methylacrylyl-CoA, tiglyl-CoA, and 3-methylcrotonyl-CoA, respectively (Fig. 3.6). These intermediates were further catabolized via different specific set of enzymes to acetyl-CoA and succinyl-CoA which are the key intermediates of TCA cycle; thus, *Pseudomonas* derive energy and carbon requirement by amino acid metabolism.

3.3.1.3 Arginine Metabolism

Arginine, a polar and positively charged amino acid, is used by pseudomonads to fulfill the requirement of polyamine (induces resistance to cationic peptide, aminoglycoside, and quinolone antibiotics) and generate ATP under energy depletion conditions. Arginine is an important nutrient and a strong chemoattractant for *P. aeruginosa*. Arginine utilization as a carbon source is restricted to some of the species of *Pseudomonas* such as *P. putida*, *P. fluorescens*, *P. aeruginosa*,

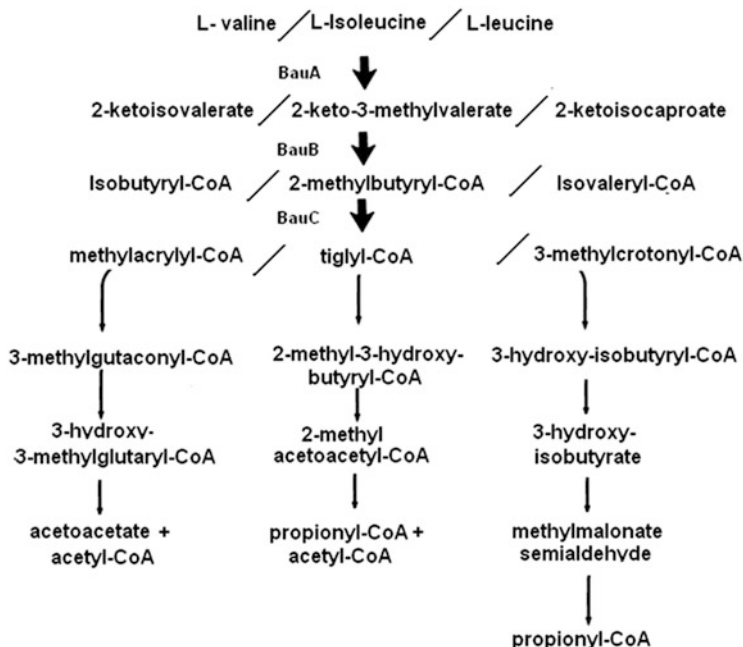


Fig. 3.6 Pathways for the metabolism of hydrophobic aliphatic branched-chain amino acids valine, isoleucine, and leucine in pseudomonads. The enzymes common in pathway are represented by wide arrows. BauA, BauB, and BauC are transaminase, keto acid dehydrogenase, and isobutyryl-coenzyme A dehydrogenase, respectively

P. mendocina, *P. stutzeri*, *P. pseudoalcaligenes*, *P. syringae*, *P. cepacia*, *P. testosteroni*, *P. acidovorans*, and *P. maltophilia* (Palleroni et al. 1974).

In *Pseudomonas aeruginosa* PAO1, the *aot* operon *aotJQMOP-argR* involved in transport of arginine and ornithine consists of six open reading frames. The sixth and terminal gene *argR* in this operon encodes ArgR, an arginine response regulator protein that controls the expression of certain genes of arginine biosynthesis and catabolism. ArgR is auto-induced by *aotJQMOP-argR* operon. Four reading frames (*aotJ*, *aotQ*, *aotM*, and *aotP*) out of six have high similarity to ABC transporters of enteric bacteria. These four genes code for proteins that function in arginine-inducible uptake of arginine and ornithine. In this process, two promoters are involved, a downstream promoter, P2, induced by arginine subject to carbon catabolite repression, and upstream promoter, P1, induced by glutamate. Both the promoters are controlled by ArgR (Nishijyo et al. 1998). ArgR protein of *P. aeruginosa* is much different in structure and function from ArgR protein of *Enterobacteriaceae* and *Bacillus subtilis*. ArgR protein also represses *argF*, *argG*, and *carAB* gene involved in arginine biosynthesis and *gdhA* and *gltBD* genes responsible for glutamate biosynthesis.

Microorganisms utilize arginine by different pathways such as (1) arginine deiminase (ADI) pathway, (2) arginine decarboxylase (ADC) pathway, (3) arginine

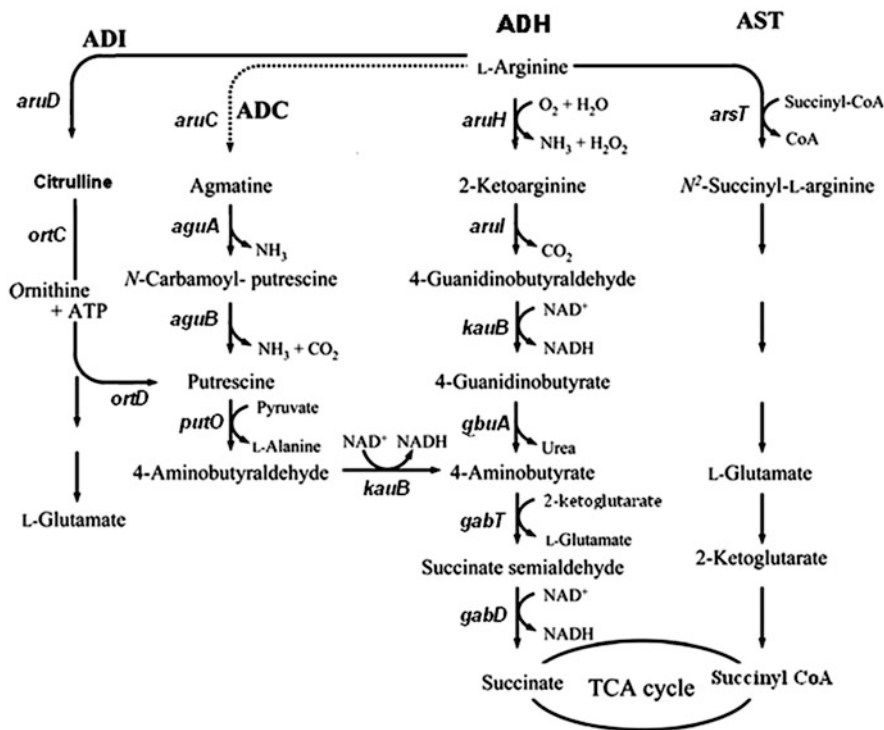


Fig. 3.7 Arginine catabolic pathways: ADI, arginine deiminase pathway; ADC, arginine decarboxylase pathway; ADH, arginine dehydrogenase pathway; AST, arginine succinyltransferase pathway; TCA, tricarboxylic acid. The enzymes (genes) involved are arginine deiminase (*aruD*), catabolic ornithine carbamoyltransferase (*ortC*), ornithine decarboxylase (*ortD*), arginine decarboxylase (*aruC*), agmatine deiminase (*aguA*), N-carbamoylputrescine hydrolase (*aguB*), putrescine oxidase (*putO*), 4-guanidinobutyraldehyde/4-aminobutyraldehyde dehydrogenase (*kauB*), arginine dehydrogenase (*aruH*), 2-ketoarginine decarboxylase (*arul*), 4-guanidinobutyrate dehydrogenase (*gbuA*), 4-aminobutyraldehyde dehydrogenase (*gabD*), 4-aminobutyrate transaminase (*gabT*), and arginine succinyl transferase (*arsT*) (Yang and Dar Lu 2007)

dehydrogenase (ADH) pathway, and (4) arginine succinyltransferase (AST) pathway (Fig. 3.7). Aerobically arginine is metabolized via AST pathway (Haas et al. 1990) and under anaerobic conditions via ADI pathway.

1. Arginine deiminase (ADI) pathway: The arginine deiminase route is a characteristic of the fluorescent group of *Pseudomonas* which serves to generate ATP under energy depletion conditions. This pathway is induced in growth conditions where culture undergoes high to low oxygen transition or depletion of carbon and phosphate sources (Mercenier et al. 1980; Stalon et al. 1982). Anaerobic regulatory protein (Aur) induces the *arcDABC* operon coding for ADI pathway enzymes and arginine/ornithine antiporter.

2. Arginine decarboxylase (ADC) pathway: The ADC pathway is a characteristic of the *Pseudomonas* strains when arginine is abundant (Palleroni et al. 1974). Much of genetic information about ADC and ADH pathway is not available. The ADC pathway may not contribute to arginine utilization due to lack of arginine-inducible ADC activity. It may rather serve to supply putrescine. Operon *aguBA* encodes agmatine deiminase (*aguA*) and *N*-carbamoylputrescine amidinohydrolase (*aguB*) converting agmatine into putrescine in this pathway. Exogenous agmatine or putrescine can induce all of the enzymes following its entry point into ADC pathway and for utilization as sole source of carbon and nitrogen. Genes for ADC pathway are not induced by arginine but arginine may be responsible for induction of an enzyme for conversion of arginine into agmatine (Haas et al. 1984; Itoh and Nakada 2004; Lu et al. 2002; Mercenier et al. 1980; Nakada et al. 2001; Nakada and Itoh 2003).
3. Arginine dehydrogenase (ADH) pathway: The arginine dehydrogenase (ADH) pathway is functional under aerobic conditions in *Pseudomonas aeruginosa* for arginine utilization. The arginine dehydrogenase also known as arginine oxidase pathway was first discovered in *Streptomyces griseus* and then in *Pseudomonas aeruginosa* and *Pseudomonas putida* (Vanderbilt et al. 1975; Van Thoai et al. 1966). In *P. putida*, at initial step, L-arginine oxidase oxidizes L-arginine to 2-ketoarginine. The *kauB* gene encodes a bifunctional enzyme having both 4-guanidinobutyraldehyde dehydrogenase and 4-aminobutyraldehyde dehydrogenase activities which transform 2-ketoarginine into intermediates 4-guanidinobutyraldehyde and 4-aminobutyrate. These intermediates are finally channeled into the tricarboxylic acid cycle through ADC pathway. Gene *kauB* is induced by 2-ketoarginine, agmatine, or putrescine and only weakly induced by D-arginine. On the other hand, 4-guanidinobutyraldehyde dehydrogenase is induced by 4-guanidinobutyraldehyde (4-GB), 2-ketoarginine, and D-arginine (Jann et al. 1988). Thus, ADH genes are regulated by distinguishable intermediates of the pathway which indicates that these genes are located at different loci on the genome. A *gbuA* mutant blocks ADH pathways and culture grows poorly when arginine was used as the sole source of carbon and nitrogen.
4. Arginine succinyltransferase (AST) pathway: The arginine succinyltransferase pathway degrades L-arginine under aerobic conditions. AST pathway is coded by operon, *aru*, and the substrate L-arginine itself induces all enzymes of this route through the mediation of arginine response regulator (ArgR) protein and generates L-glutamate and succinate as end products.

Thus, all the arginine catabolic pathways ultimately enter the TCA via succinate which allows *Pseudomonas* to derive energy and carbon requirement by amino acid metabolism.

3.3.1.4 Lysine Metabolism

Lysine a basic amino acid is abundantly available in rhizosphere; its both D- and L-forms are prevalent in the biological system which serves as a carbon and nitrogen source for pseudomonads. The conversion of L-lysine to glutarate represents the

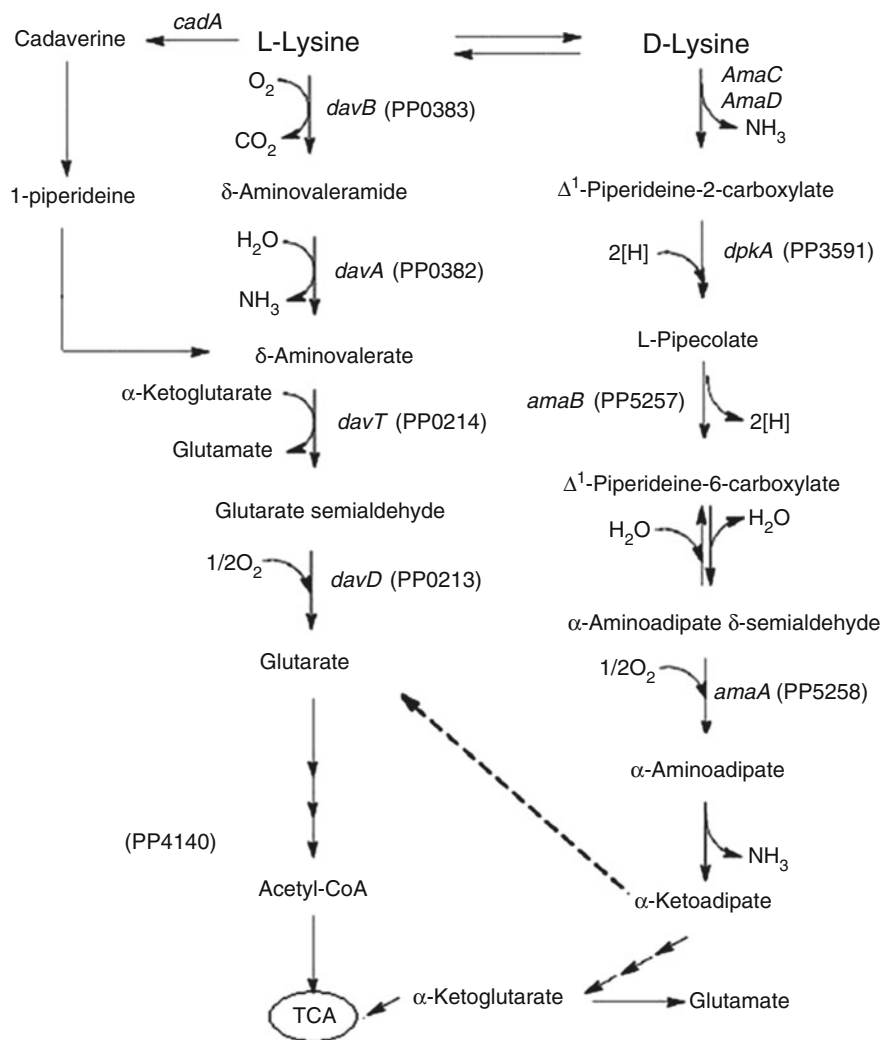


Fig. 3.8 Catabolic pathways for the degradation of L- and D-lysine by genus *Pseudomonas*: The conversion of L-lysine to glutamate represents the aminovaleramide (AMV) pathway, and D-lysine to aminoadipate represents the aminoadipate (AMA) pathway, and the left branch of two reactions from L-lysine to δ -aminovalerate via cadaverine/1-piperidine represents the cadaverine pathway. The corresponding genes of translated product are given

aminovaleramide (AMV) pathway and the conversion of D-lysine to aminoadipate represents the aminoadipate (AMA) pathway (Fig. 3.8). The pathways are inducible by D-lysine and L-lysine, respectively, and enzyme racemase for their interconversion has been reported (Soda and Moriguchi 1969). L-Lysine is primarily utilized by AMV pathway, leading to the formation of α -ketoglutarate that enters TCA cycle (Revelles et al. 2005).

In AMV pathway (a monooxygenase pathway), four genes *davABDT* have been identified. Genes *davDT* for metabolism of δ -aminovalerate (δ -AMV) to glutarate form one operon, and *davBA* for L-lysine to δ -AMV form second operon. Conversion of D-lysine to Δ' -piperidine-2-carboxylate is mediated by two enzymes, viz., D-lysine-amino transferase (*AmaC*) and D-amino acid dehydrogenase (*AmaD*) encoded by ORF 3590 and ORF 3596. D-amino acid dehydrogenase oxidized D-lysine into 6-amino-2-oxohexanoate (2-Keto-6-amino-caproic acid), which gets converted spontaneously into Δ' -piperidine-2-carboxylate. Likewise, conversion of L-pipecolate to 2-aminoadipate, genes *amaB* and *amaA*, form a different operon. Pipecolate is metabolized to α -ketoglutarate through the formation of 2-amino- α -adipate and 2-ketoadipate. Several *P. putida* strains can use D-lysine as carbon and nitrogen involving a parallel pathway (AMA) as shown in Fig. 3.8, and genes for this pathway have been reported to be coded on OCT (octane) plasmid of *P. putida* which normally encodes enzymes required for degradation of octane (Muramatsu et al. 2005; Revell et al. 2007).

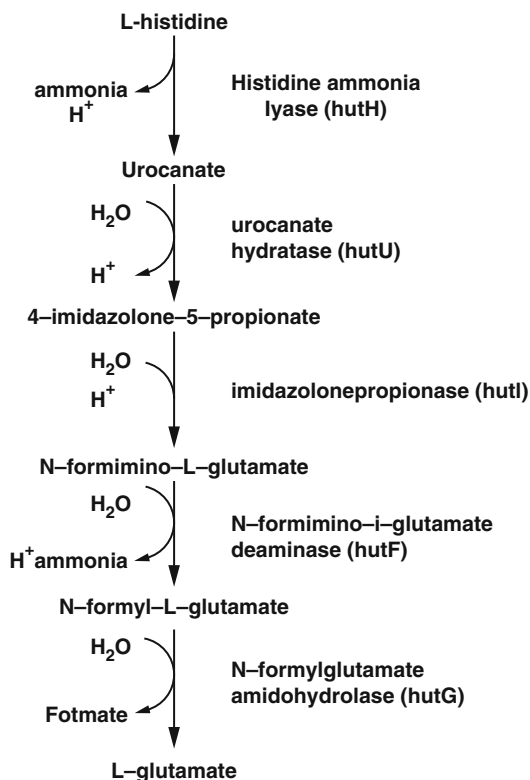
Apart from monooxygenase pathway, lysine catabolism can be initiated through decarboxylase or a transaminase pathway. For *P. putida*, monooxygenase is the main route and genes *davABDT* code the first four steps of the pathway. However, *P. aeruginosa* grows poorly on exogenous lysine and lack *davA* and *davB*, and thus the first two steps of AMV pathway (a monooxygenase pathway) are absent but *cadA* gene and pipecolate pathway are present. In *P. syringae* *cadA* gene is absent although genes of AMV and AMH pathways are present, while *P. fluorescens* PAO-1 possesses both gene *cadA* and genes for the pathways AMV and AMA. Alternatively, conversion of lysine into aminovalerate in *P. aeruginosa* can also be carried out by arginine-pyruvate transaminase and 2-ketoarginine decarboxylase coded by *aruH* and *aruI*, respectively. Transamination of lysine can yield α -keto- ϵ -aminohexanoate which can then be decarboxylated to 5-aminovalerate by AruI protein. Thus, L-lysine is metabolized by more than one pathway as indicated by simultaneous accumulation of three products, viz., δ -aminovalerate, pipecolate, and aminoadipate (Revell et al. 2007).

3.3.1.5 Histidine Metabolism

Degradation of histidine has been established in *P. aeruginosa*, *P. putida*, *P. testosterone*, and *P. fluorescens*. The first step is deamination by histidine-ammonia lyase to form urocanic acid. The pathway is similar to one that operates in *Salmonella typhimurium* and *B. Subtilis* (Leidigh and Wheelis 1973; Coote and Hassall 1973).

Urocanase, a photoactivated enzyme, forms imidazolone- propionic acid. In the next step, enzyme hydrolase forms a branched-chain molecule, formiminoglutamic acid. Enzyme formiminoglutamase forms glutamate and formamide (Fig. 3.9). These five steps are coded by *hutHUIFG* operon (Leidigh and Wheelis 1973). Glutamic acid transaminase forms α -ketoglutaric acid that enters TCA cycle. Formamide is deaminated to form formic acid for further metabolism. Histidine metabolism pathway is subject to induction and repression. Hug et al. (1968) reported that succinate exerts sequential feedback inhibition of urocanase and

Fig. 3.9 Catabolic pathway of histidine by *Pseudomonas putida* KT2440 controlled by HutH (histidinase) HutU (urocanase), HutI (imidazolone propionate hydrolase, IPAase), HutF (N-formimino-L-glutamate deiminase, FIGLUase), and HutG (formylglutamate amidohydrolase, FGase)



then urocanic acid inhibits histidine lyase. Urocanic acid also acts as an inducer in *Salmonella typhimurium*, while in *B. subtilis* histidine acts as an inducer for “hut” enzymes.

Nonfluorescent pseudomonads, *P. testosteroni* and *P. acidovorans*, are able to grow on imidazolyl-propionate or imidazolyl-lactate. Imidazolyl-lactate forms histidine through imidazolyl-pyruvate, while imidazolyl-propionate forms urocanate. These imidazolyl derivatives are not metabolized by *P. aeruginosa* and *P. putida*.

Five-step histidine degradation is controlled by *hut* operon comprising of *hutH* (histidinase), *hutU* (urocanase), *hutI* (imidazolone propionate hydrolase, IPAase), *hutF* (N-formimino-L-glutamate deiminase, FIGLUase), and *hutG* (formylglutamate amidohydrolase, FGase) (Hu and Phillips 1988). In *P. testosteroni* and *P. putida*, all enzymes are induced by urocanate, while FGase is induced by its substrate formylglutamate as well (Kaminskas et al. 1970). In *P. putida*, the *hut* genes are located on a 16 kb segment as determined by cloning in *E. coli* which lacked the ability to dissimilate histidine (Fig. 3.10).

Ecological and metabolic diversity of *Pseudomonas* has been related to regulatory system that coordinates the response to the environment. That is why

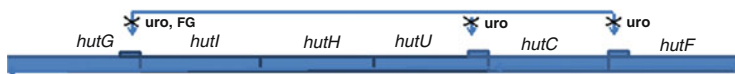


Fig. 3.10 *Hut* operon comprising of *hutG* (formylglutamate amidohydrolase, FGase), *hutI* (imidazolone propionate hydrolase, IPAase), *hutH* (histidinase), *hutU* (urocanase), *hutC* (histidine utilization repressor), and *hutF* (N-formimino-L-glutamate deiminase, FIGLUase). The expression of the genes is regulated by urocanate (uro) and formylglutamate (FG)

Pseudomonas possesses a high number of regulatory systems. In *P. putida* KT2440, it has been estimated that nearly 1/10th of the genome encodes products involved in signal transduction and gene regulation. High numbers of transcriptional regulatory proteins responsible for the functional versatility have been reported.

A two-component system *CbrAB* has been described as global regulatory system for assimilation of histidine, proline, and arginine in *Pseudomonas*. The CbrA is a sensor histidine kinase containing transmembrane domains. In *P. aeruginosa*, the CbrB (a cognate response regulator) system is required for growth on different nutrients including amino acids and other nitrogen-containing substrates as sources of carbon (Nishijyo et al. 2001). CbrB is also an activator of σ^{54} -dependent promoters belonging to NtrC family (regulatory proteins that participate in the transduction of extracellular and nutritional signals). The σ^{54} along with *hutC* and urocanate regulates *hutU-G*.

The CbrAB system is directly required for expression of arginine and histidine catabolic genes. The suppression of the defect of CbrAB mutants by constitutive *ntrB* and *ntrC* alleles indicates that some of the targets are under dual CbrAB/NtrBC control (Li and Lu 2007). Besides *P. aeruginosa*, this type of system has been observed in *P. fluorescens* and *P. putida* KT2440. CbrB-dependent regulation of genes is related to carbon metabolism, amino acid uses, polyamines, and some unrelated functions such as chemotaxis, tolerance to metals, and other stress responses. CbrB may be important for adaptability of the *Pseudomonas* to changing environment (Zhang and Rainey 2007).

Complexity of *hut* regulation suggests that the metabolism of histidine in *Pseudomonas* is of key importance. HutC repressor along with urocanase, which is the first intermediate of the histidine degradation pathway, negatively regulates the *hut* operons. The negative regulation indicates that *hut* operon is not frequently used and that histidine is scarce in natural environment. The scarcity of histidine has been shown in the rhizosphere (Zhang et al. 2006). On the contrary the positive control by CbrB shows that histidine is frequently encountered in the environment. Therefore, Zhang and his group have suggested that CbrAB has a more general role such as a sensor for a range of amino acids and as a positive activator for a number of specific degradation pathways.

3.3.1.6 Proline Metabolism

Proline utilization requires two divergently transcribed genes *putP* and *putA* which form a single *put* operon (Hechtman and Scliver 1970). The *put* operon is induced by proline, whereas PutA protein is involved in regulation of *put* operon. In the

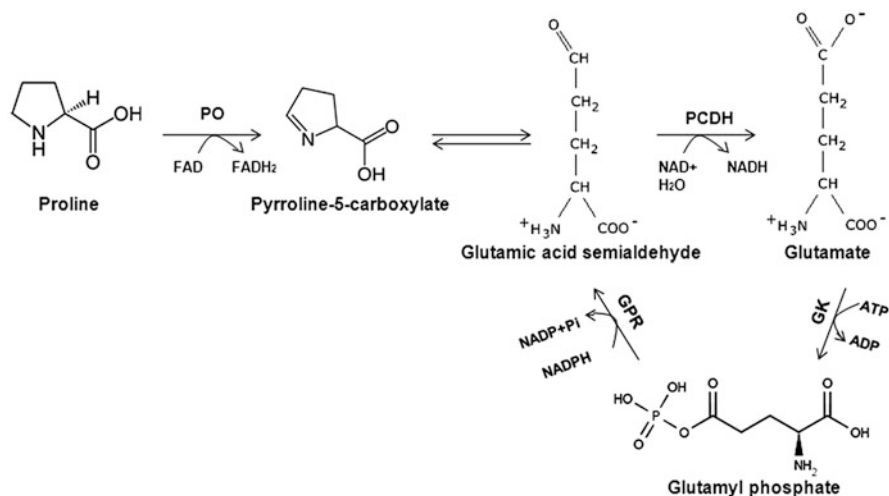


Fig. 3.11 Pathway of proline catabolism in pseudomonads: The enzymes catalyzing successive reactions are as follows: proline oxidase (PO) and pyrroline-5-carboxylate dehydrogenase (PCDH) coded by *putA* operon which catabolize proline to glutamate a compound for TCA. Glutamate may be converted to glutamic acid semialdehyde via glutamyl phosphate by the use of enzymes glutamyl kinase (GK) and glutamyl phosphate reductase for generation of NADPH

presence of proline, the PutA protein becomes membrane associated and the *put* genes are fully expressed. The *putP* gene encodes a major proline permease and *putA* shows two distinct enzymatic activities: proline oxidase and pyrroline-5-carboxylate dehydrogenase (Maloy 1989) (Fig. 3.11). Proline oxidase reaction couples proline oxidation to reduction of FAD cofactor. The re-oxidation of the reduced FAD requires association of the enzyme with electron transport chain in the membrane. Therefore, PutA protein must be membrane associated in presence of proline.

In the absence of proline, the PutA protein may remain in cytoplasm where it is able to bind the operator site between *putA* and *putP* promoters, thus repressing the expression of both genes. Menzel and Roth (1981) and Ostrovsky de Spicer et al. (1991) confirmed these observations and suggested that PutA protein may accumulate in the cytoplasm and bind to multiple sites in the *put* control region. In the presence of proline as an inducer, proline oxidation would reduce tightly associated FAD coenzyme, inducing PutA protein to become membrane associated and abandon the operator sites, thus allowing expression of the *put* genes. Thus, PutA protein functions as enzyme that catalyzes two different catabolic steps and as a repressor by binding at multiple sites.

P. putida KT2440 utilizes proline as a source of carbon and nitrogen. Gene cloning analysis show that, in *P. putida*, the process of proline utilization is analogous to enteric bacteria involving a *putP* gene for uptake of proline and *putA* gene coding for a multifunctional protein catalyzing two-step transformation of proline into glutamate (Vilchez et al. 2000). PutA protein of *P. putida* KT2440 is

a 1315-amino acid residue protein and bears homology with PutA protein of *E. coli*, *S. typhimurium*, *Rhodobacter capsulatus*, and several strains of *Rhizobium*. PutP protein of *P. putida* is 493-amino acid long and shows 85 % similarity with PutP of *P. fluorescens*, 76 % with *Salmonella typhimurium*, and 78 % with that of *E. coli*.

3.3.1.7 Tyrosine and Phenylalanine

Pseudomonas putida catabolizes phenylalanine and tyrosine through a peripheral pathway involving hydroxylation of phenylalanine to tyrosine and then conversion of tyrosine to 4-hydroxyphenylpyruvate and formation of homogentisate as an intermediate for the central pathway.

In the first step, pterin-dependent phenylalanine hydroxylase (*phhA*) catalyzes the conversion of phenylalanine to tyrosine. This also involves another enzyme carbinolamine dehydratase (*phhB*) which catalyzes regeneration of pterin cofactor. This is under the regulatory control of transcriptional activator (*phhR*) of the *phh* operon *phhRAB*. These genes are homologous to the genes characterized in *P. aeruginosa* and *P. fluorescens*.

In *Pseudomonas putida* KT2440, the *phhRABT* cluster carries a gene encoding a transport protein (*phhT*) close to the gene *aroP₂* coding a general permease for aromatic amino acids. In *P. aeruginosa*, gene *phhC* codes for tyrosine-amino-transferase catalyzing the transformation of tyrosine into 4-hydroxyphenylpyruvate which is an essential step in catabolism of phenylalanine and tyrosine. In *P. putida*, no homologue of *phhC* has been detected, but two other genes *tyrB₁* and *tyrB₂* encode tyrosine aminotransferase (TyrB). The *tyrB* genes are not linked to *phhRAB* operon in *P. putida* and *P. syringae*. This organization is similar to other organisms such as *Azotobacter vinelandii*, *Xanthomonas axonopodis*, and *R. solanacearum* (Jiménez et al. 2002). 4-Hydroxyphenylpyruvate through series of enzymatic steps involving HmgA (homogentisate dioxygenase), HmgC (maleylacetoacetate isomerase), and HmgB (fumarylacetoacetate hydrolase) gets converted into fumarate which is an important intermediate of TCA cycle (Fig. 3.12).

PhhR gene product has been implicated as a transcriptional regulator that is critical for induction of phenylalanine/tyrosine catabolic genes. *PhhR* mutants are unable to grow or grow poorly in phenylalanine and tyrosine; however, the growth with tryptophan was not affected. *PhhR* modulates transcription from σ^{54} - and σ^{70} -dependent promoters, and their binding sites have been identified between *PhhR* and *PhhA* (Palmer et al. 2010).

Phenylalanine and tyrosine are important nutrient for *Pseudomonas* in sputum. The ability of *Pseudomonas aeruginosa* to metabolize phenylalanine as both carbon and nitrogen source allow growth of this opportunistic pathogen in human lungs. The abundant availability of aromatic amino acids caused feedback inhibition of the biosynthesis of these amino acids, thus allowing precursor metabolites to be fluxed toward *Pseudomonas* quinolone signal (PQS). The PQS regulates production of toxic factor which includes hydrogen cyanide, elastase, and pyocyanin which lead to cystic fibrosis lung (CFL). *Pseudomonas aeruginosa* produces approximately fivefold more PQS in the presence of aromatic amino such as tryptophan or a mixture of tryptophan, phenylalanine, and tyrosine (Palmer et al. 2005).

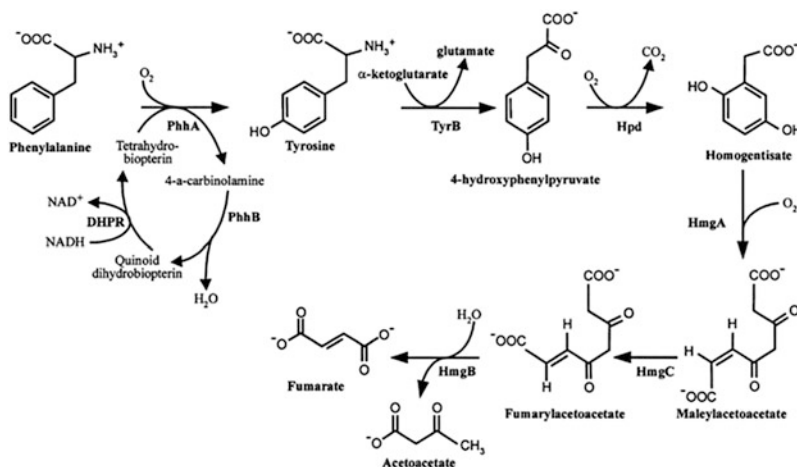


Fig. 3.12 Pathway for the catabolism of phenylalanine and tyrosine in *P. putida*: The enzymes are PhhA (phenylalanine hydroxylase); PhhB (carbinolamine dehydratase) for initial conversion of phenylalanine to tyrosine and regeneration of cofactors, respectively; TyrB (tyrosine aminotransferase); Hpd (4-OH-PhPy dioxygenase); HmgA (homogentisate dioxygenase); HmgB (fumarylacetoacetate hydrolase); HmgC (maleylacetoacetate isomerase); and dihydropteridine reductase (DHPR)

3.3.1.8 Tryptophan Metabolism

Tryptophan is a biosynthetic precursor of cofactors (NAD), antibiotics, siderophores, etc. Certain bacteria have the enzymatic system to catabolize tryptophan and utilize it as sole source of carbon and nitrogen. Pseudomonads can catabolize tryptophan by four different pathways classified as:

1. Aromatic group, degrades L-tryptophan via anthranilic acid
2. The quinoline group, degrades D- and L-tryptophan via kynurenic acid
3. The racemase-aromatic group, degrades D- and L-tryptophan via anthranilic acid
4. Quinazoline group, degrades D- and L-tryptophan via O-aminoacetophenone

The aromatic group is represented by *P. fluorescens* and attacks the tryptophan by L-specific tryptophan-2,3-dioxygenase (L-tryptophan/oxygen-2,3-oxidoreductase). The tryptophan dioxygenase containing a heme as prosthetic group inserted oxygen molecule into pyrrole ring and converts L-tryptophan to L-formylkynurenine (Fig. 3.13).

Then kynurenine formamidase (aryl-formylamine amidohydrolase) splits formyl-L-kynurenine into kynurenine and formic acid. Enzyme kynureninase (L-kynurenine hydrolase) hydrolyzes the side chain of kynurenine forming anthranilic acid and alanine. Oxidative decarboxylation and deamination of anthranilic acid form catechol, which is a key intermediate in the metabolism of aromatic compounds.

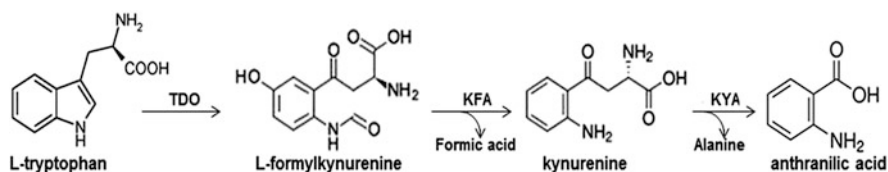


Fig. 3.13 Pathway of tryptophan catabolism by aromatic group, which degrades L-tryptophan via anthranilic acid. Enzymes involved are TDO (tryptophan-2,3-dioxygenase), KFA (kynurenine formamidase), and KYA (kynureninase)

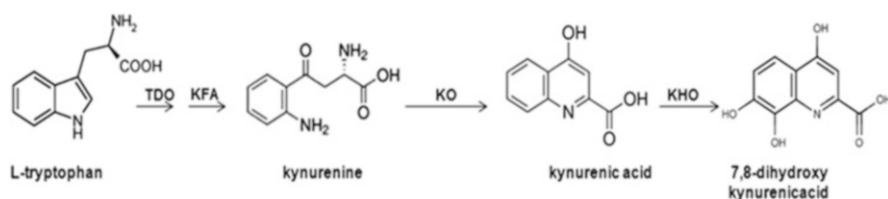


Fig. 3.14 Pathway of tryptophan catabolism by quinoline group, which degrades D- and L-tryptophan via kynurenic acid. Enzymes involved are TDO (tryptophan-2,3-dioxygenase), KF (kynurenine formamidase), and KHO (kynurenate 7,8-hydroxylase)

Second pathway involves quinoline group in *P. acidovorans* which catabolizes L- and D-tryptophan via kynurenic acid by respective oxygenase and kynurenine formamidase to D- or L-kynurenine. *Pseudomonas acidovorans* uses L- or D-kynurenine oxidase to oxidize kynurenine to kynurenic acid. Then a hydroxylase (kynurenate 7,8-hydroxylase) converts kynurenic acid to 7,8-dihydroxy kynurenic acid which further by series of reactions converted into 2-oxoglutarate and oxaloacetate (Fig. 3.14).

The third, the racemase group, degrades D- and L-tryptophan via anthranilic acid. In this pathway, the D-tryptophan is first converted into L-isomer by the racemase, and then catabolism proceeds as in the case of *P. fluorescens* via anthranilic acid.

The fourth pathway involves the quinazoline group represented by *P. aeruginosa* and metabolizes D- and L-tryptophan via O-aminoacetophenone. The metabolites of this pathway are identified but there are no reports available so far regarding enzymes involved in this pathway.

Catabolic pathway of tryptophan by pseudomonads differs from that of eukaryotes probably because of the involvement of an oxygenase. Tryptophan degradation also occurs in other genera such as *Streptomyces*, *Bacillus*, *Flavobacterium*, and *Burkholderia*.

In the kynurenine pathway for NAD biosynthesis, tryptophan catabolic pathway splits at 2-amino-3-carboxymuconate semialdehyde (ACMS) (Fig. 3.15). ACMS is converted into quinolinic acid non-enzymatically as a precursor of NAD or ACMS decarboxylase (ACMSD) forms 2-aminomuconate semialdehyde (AMS) for further conversion into an 2-aminomuconate by a dehydrogenase (AMDH).

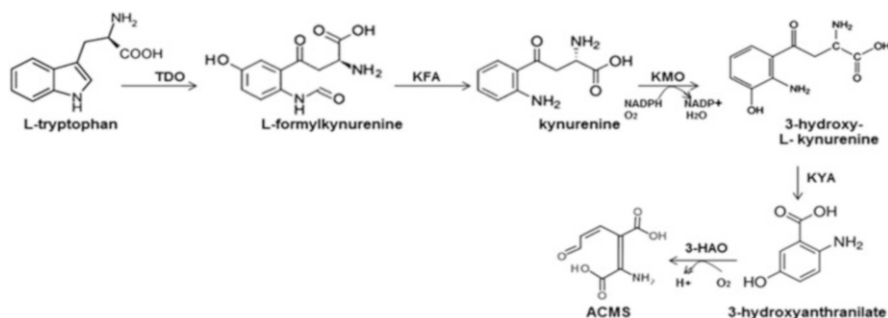


Fig. 3.15 Tryptophan catabolic pathway for synthesis of 2-amino-3-carboxymuconate semialdehyde (ACMS) which is a major intermediate kynurenine pathway for NAD biosynthesis. A set of five enzymes involved are TDO (tryptophan-2,3-dioxygenase), KFA (kynurenine formamidase), kynurenine-3-monooxygenase, KYA (kynureninase), and 3-HAO (3-hydroxyanthranilate oxidase)

Discovery of a set of five genes for synthesis of ACMS from tryptophan suggests that similar pathway operates in prokaryotes and eukaryotes. Comparison of the genetic data on catabolic gene clusters of tryptophan for 3-hydroxyanthranilate-3,4-dioxygenase (HAD) and ACMSD were identified. In *Burkholderia cepacia* J2315, HAD and ACMSD orthologs occurred in clusters of genes of unknown function. Sequence analysis indicated that one of these genes may function as 2-aminomuconate semialdehyde dehydrogenase (AMHD) and another as 2-aminomuconate deaminase (AMD).

Another cluster lies just upstream of the HAD–AMD cluster. This comprised of known homologues of 4-oxalocrotonate decarboxylase (4OCD), 2-ketopentanoate hydratase (KPH), 2-keto-4-hydroxypentanoate aldolase (HOA), and acetaldehyde dehydrogenase (ADH). Three other genes encoding tryptophan-2,3-dioxygenase (TDO), kynurenine formamidase (KFA), and kynureninase (KYN) were also identified in this cluster, while no kynurenine-3-monooxygenase (KMO) was identified. This suggested the existence of second non-orthologous KMO in *Burkholderia*. All the eight genes were earlier identified within the uninterrupted cluster in *B. cereus*. In the new pathway in *B. cepacia* J2315, the 2-aminomuconate is deaminated to 4-oxalocrotonate rather than 2-ketoadipate earlier proposed for mammalian cells. This was further strengthened by PCR amplification of putative HAD, ACMSD, AMDH, and AMD genes, cloning in pDESTFI and expressed in *E. coli*.

In *P. fluorescens* and *P. acidovorans*, the first two enzymes tryptophan pyrrolase and formylkynurenine formamidase are coordinately induced by L-kynurenine. Both these enzymes are present at low concentration in resting cells so that the inducer is generated endogenously. Anthranilic acid and kynurenic acid do not induce these enzymes though they are the inducers for the synthesis of all the enzymes involved in the subsequent pathway (Palleroni and Stanier 1964; Rosenfelh and Feigelson 1969). L-Kynurenine also induces synthesis of

kynureninase, but its synthesis is not coordinated with tryptophan pyrrolase and formamidase. L-Tryptophan does not induce any of these enzymes.

3.3.2 Metabolism of Alkanes

Alkanes are saturated hydrocarbons, i.e., exclusively comprising of carbon and hydrogen atoms. Alkanes are abundantly available in nature as these constitute about 50 % of the crude oil. These are also generated by biota present in environment such as plants, animals, algae, and bacteria and thus found in low concentration in soil and water as well. Their additional load to soils and water systems by accidental spills and leaks during refining, transport, and storage of petroleum products is a serious environmental concern. In general, alkanes fall in three groups, the linear (*n*-alkane), cyclic (cyclo-alkanes), and branched (iso-alkanes). Low molecular weight alkanes C₁–C₄ are gaseous and miscible with water, medium-chain length C₅–C₁₆ are liquids, and ≥C₁₇ are solids. These molecules have very low solubility in water because alkanes are chemically inert at room temperature due to presence of four sigma bonds which requires high temperature to break. Thus, inertness and low solubility of alkanes makes their uptake difficult by indigenous microbial cells (Wentzel et al. 2007). This also results in accumulation of alkanes in the membrane of the cells and adversely affects membrane fluidity and function.

Certain microorganisms including *Pseudomonas* have acquired ability to metabolize alkanes as carbon and energy sources (van Beilen et al. 2003; Wentzel et al. 2007). Rosenberg (1991) estimated that typically a sample of soil, sand, or ocean sediment contains 10⁴–10⁶ hydrocarbon-degrading microorganisms per gram, and these values could be higher for oil-polluted sites. *Pseudomonas* grows aerobically on C₂–C₄ gaseous alkanes but not on methane. Best studied among these are *Pseudomonas butanovora*, *P. putida* GPoI (*P. oleovorans* GPoI), and *P. aeruginosa*. However, the precise mechanism of alkane uptake is not yet clear (Wentzel et al. 2007). The low molecular weight alkanes which are sufficiently soluble in water may be taken up directly from water phase. For the medium- and long-chain *n*-alkanes, microorganisms may gain access either through adhering to the hydrocarbon droplets or by surface-active agent that bacteria are known to produce.

Bacteria capable of degrading *n*-alkane are known to produce surface-active molecules, known as biosurfactants, that help in the emulsification of hydrocarbons (Hommel 1990; Ron and Rosenberg 2002). Biosurfactants act by increasing the surface area of the hydrophobic molecule to water phase and thus facilitating access to microorganisms. In liquid medium cultures, surfactants have been reported to improve growth of cultures by faster utilization of alkanes. They also protect bacterial cells from direct exposure to toxic compounds (Kang and Park 2009).

3.3.2.1 Aerobic Degradation of Alkanes

Microorganisms have the ability to degrade inert compounds like saturated alkanes by inserting an oxygen atom into a carbon–hydrogen bond. In the first step, enzyme monooxygenase inserts an oxygen atom into the terminal methyl group of alkanes to form a primary alcohol, which is further oxidized to corresponding aldehyde and organic acid by specific alcohol and aldehyde dehydrogenases. The fatty acid thus formed is conjugated with coenzyme A (CoA) and undergoes β -oxidation to generate acetyl-CoA (Wentzel et al. 2007).

Some microorganisms also carry out subterminal oxidation of *n*-alkanes forming secondary alcohol which is converted to a ketone (Whyte et al. 1998; Kotani et al. 2006; Kotani et al. 2007). The enzyme Baeyer–Villiger monooxygenase (BVM) oxidizes the ketone to form an ester, and finally enzyme esterase splits it into alcohol and fatty acid moieties (Fig. 3.16). The oxidation of fatty alcohols and fatty acids is common in microorganisms but the hydroxylases required for

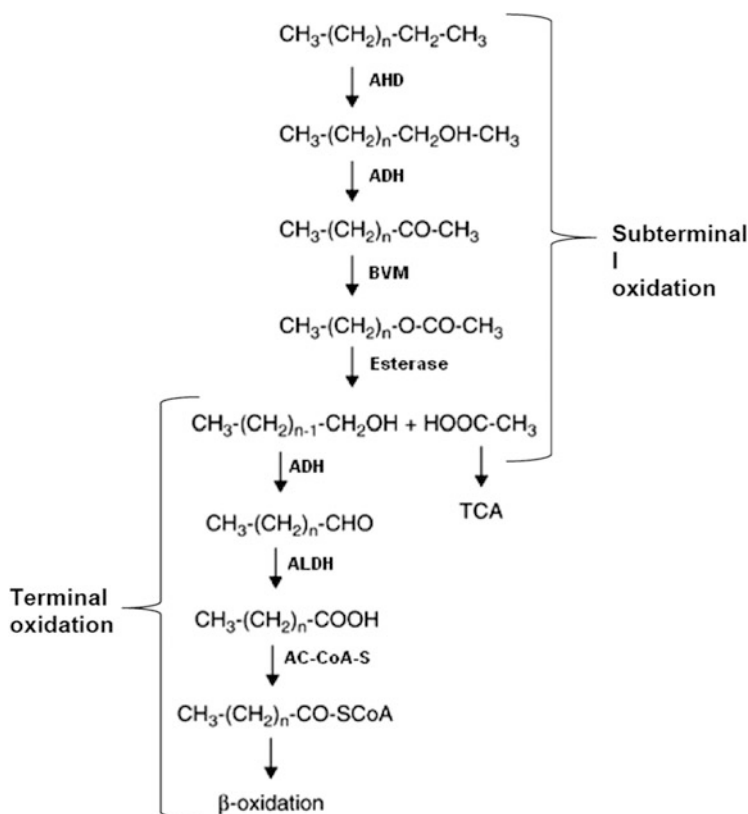


Fig. 3.16 Aerobic degradation of alkanes by *Pseudomonas*: Subterminal oxidation involving alcohol hydratase (AHD), alcohol dehydrogenase (ADH), Baeyer–Villiger monooxygenases (BVM), and esterase. Terminal oxidation of alcohols to fatty acids carried out by enzymes alcohol hydratase (AHD), aldehyde dehydrogenase (ADH), and acetyl-CoA synthetase

oxidation of alkanes are much less wide spread. Bacteria degrading short-chain alkanes (C_2 – C_4) have enzymes related to methane monooxygenases (Hamamura et al. 1999; Dubbels et al. 2007), whereas strains degrading medium-chain-length alkanes (C_5 – C_{11}) or long-chain alkanes ($>C_{12}$) generally contain membrane-bound non-haem iron monooxygenase.

Pseudomonas butanovora can assimilate C_2 – C_4 alkanes by sequential oxidation of terminal CH_3 group of the hydrocarbon by alcohol inducible butane monooxygenase (BMO), a broad substrate range alkane monooxygenase. The enzyme has three subunits: (1) a dinuclear iron containing butane monooxygenase (BMOH) that comprises of three polypeptides, (2) an NADH-oxidoreductase (BMOR), and (3) a small regulatory protein (BMOB) that acts as an effector, which may be partly dispensable (Dubbels et al. 2007). Kurth et al. (2008) reported that proper assembly of BMO requires chaperonin-like protein Bmo G. On the 5' end of BMO operon lies a putative σ^{54} -dependent promoter which is subject to positive control by enhancer-binding proteins that facilitate initiation of transcription (Fig. 3.17). Based on the current understanding of the BMO, operon following model has been proposed for regulation of butane metabolism by *P. putida* GPoI.

P. putida GPoI contain plasmid for degradative assimilation of alkane. The first enzyme is membrane-bound non-haem di-iron monooxygenase (Alk B) that hydroxylates alkane at the terminal position. This requires two soluble electron transfer proteins rubredoxin (Alk G) and rubredoxin reductase (Alk T). Alk T transfers the electrons from NADH to rubredoxin via cofactor FAD, which then transfers the electrons to Alk B. Alk B has six transmembrane segments and a catalytic site that faces the cytoplasm. Active site includes four histidine-containing sequence motives that are conserved in other hydrocarbon monooxygenases and chelates two iron atoms. Di-iron cluster allows oxygen-dependent activation of alkane molecule through substrate radical intermediate. One of the oxygen atoms is transferred to the terminal methyl group of the alkane, thus converting it into an

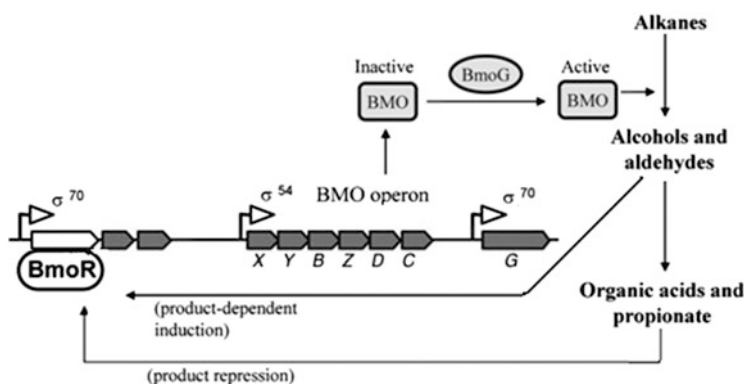


Fig. 3.17 BMO operon regulation by an NADH-oxidoreductase (BmoR) which itself induced and repressed by alcohols/aldehydes and organic acids/propionates, respectively. Proper assembly and activation of BMO require chaperonin-like protein BmoG

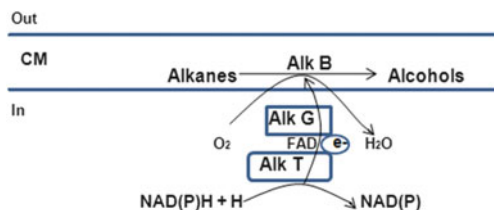


Fig. 3.18 Degradation of alkane by membrane-bound non-haem di-iron monooxygenase (Alk B) using electron transfer proteins rubredoxin (Alk G) and rubredoxin reductase (Alk T). Alk T transfers the electrons from NADH to rubredoxin via cofactor FAD, which then transfers the electrons to Alk B

alcohol, while the second atom of oxygen is reduced to H₂O by electrons transferred by rubredoxin (Fig. 3.18). *P. putida* GPoI Alk B alkane hydroxylase can oxidize propane and butane (Johnson and Hyman 2006) as well as C₅–C₁₃ alkanes (Van Beilen et al. 2005). All these support the growth of *P. putida* GPoI, but methane, ethane, and alkanes longer than C₁₃ are not oxidized.

However, in *P. aeruginosa* PAO1, there are two alkane hydroxylases, viz., Alk B1 alkane hydroxylase oxidizes C₁₆–C₂₄ *n*-alkanes and Alk B2 alkane hydroxylase is active against C₁₂–C₂₀ *n*-alkanes. These two enzymes have overlapping substrate specificity and are not induced simultaneously (Marín et al. 2001). A number of gram-positive and gram-negative bacteria carry Alk B alkane hydroxylase, and more than 60 Alk B homologues have been identified, but they show high sequence diversity.

Rubredoxin is small redox-active iron–sulfur protein and transfers electrons to Alk B. The Alk G rubredoxin of *P. putida* PoI is unusual as it contains two rubredoxin domains. Alk G1 and Alk G2 linked through a linker, while rubredoxins from other microorganisms have only one domain. Rubredoxin–rubredoxin reductase systems have also been reported in other organisms that do not degrade alkanes and serve other functions such as oxidative stress responses in anaerobic bacteria (Frazao et al. 2000). Rubredoxin–rubredoxin reductase complex in *P. aeruginosa* is responsible for rapid transport of reducing equivalents to final receptor (Hagelueken et al. 2007).

P. aeruginosa AT18 isolated from the site of a refinery in Cuba contaminated with crude oil was able to grow on kerosene (C₁₂–C₁₄), lubricant oil (C₁₈–C₄₀), toluene (alkylbenzene), and naphthalene. *P. aeruginosa* AT18 has also been reported to grow on crude oil (M30PP) having density of 0.835 mg l⁻¹ and API gravity of 26.2. The composition of M30PP is *n*-paraffin 23.9%, iso-paraffins 26.1%, naphthalene 25.7%, and aromatics 24.3% (Silva et al. 2006). From a similar site, Liu et al. (2011) have reported the isolation of *P. aeruginosa* SITD-1 that is capable of utilizing long-chain alkanes (*n*-hexadecane, *n*-octadecane, *n*-tetradecane, and *n*-triacontane) as sole source of carbon and energy, and besides it can also use diesel oil and crude oil efficiently.

The enzyme systems described above have been discovered recently and only limited studies have been undertaken about their prevalence in other organisms in the environment. Genes almost identical to Alk B of *P. putida* GPoI were found in *P. putida*, *P. aeruginosa*, and *P. mendocina*. Alkane hydroxylases associated with soluble cytochromes P450 prevalent in *Acinetobacter* sp. have not been detected in *Pseudomonas* although prevalent in *Rhodococcus*, *B. megaterium*, and *Candida apicola* strains.

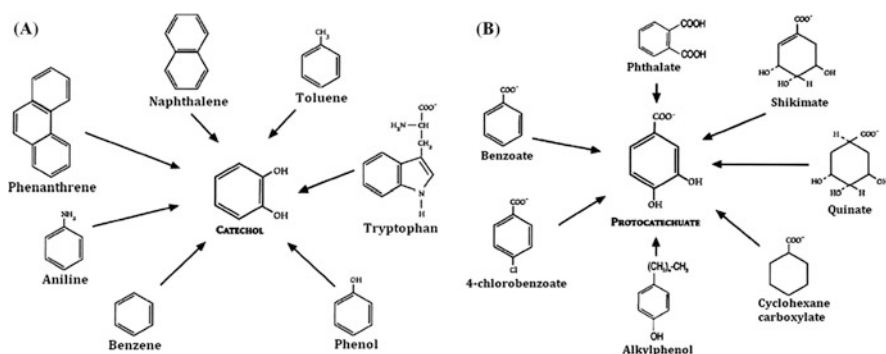
Diversity of the hydroxylase genes and coexistence of different hydroxylases in the same organisms indicate that they might have evolved through horizontal transfer of genetic material. Phylogenetic analysis of 58 Alk B-related proteins identified in different gram-positive and gram-negative bacteria showed that Alk B homologues from fluorescent pseudomonads were almost as divergent as the entire set of genes analyzed. Alkane degradation genes have been found in transposons and plasmids which further support the contention of horizontal gene transfer (van Beilen et al. 2003).

3.3.3 Degradation of Aromatic Hydrocarbons

The industrially produced aromatic compounds (e.g., styrene, toluene, benzene, etc.) are mostly potential xenobiotic and pose a threat to living organisms. These are known to be degraded slowly by microorganisms due to factors such as stress response to substrate toxicity, substrate-dependent induction, catabolite repression, etc. However, in recent years, apart from ability of *Pseudomonas* to cause disease in humans, their role emerged in detoxification of chemical wastes which involves breakdown of xenobiotic organic compounds to water and less toxic intermediates (Singh et al. 2009; Golovleva et al. 1992). The degradation of such complex organic molecules requires a sequential working of different enzymes. The enzymes of peripheral metabolism involved in the primary attack on xenobiotics have a broad substrate specificity in *Pseudomonas* as these are capable of converting not only their substrate but also its structural analogs, e.g., toluene dioxygenase responsible of the transformation of toluene and also trichloroethylene, *p*-dichlorobenzene, phenol, and 2,5-dichlorophenol (Wackett and Gibson 1988; Zylstra et al. 1988). The genes coding for these enzymes may be located in the chromosomal DNA but very often found on plasmids. The genetic information, which is required for the survival of pseudomonads in the environment low in nutrients, flow among indigenous microorganisms on plasmids, conferring new degradative potential in microbial strains for bioremediation (Fulthorpe and Wyndham 1991; Van der Meer et al. 1991). There are many plasmids reported in pseudomonads which confer metabolic diversity for degradation of aromatic hydrocarbons, e.g., TOL plasmid of 117 kb contains genes to degrade xylene, toluene, and toluate; NAH7 of 83 kb metabolize naphthalene via salicylate; pKF1 of 82 kb have genes for degradation of biphenyls via benzoate; pWW174 of 200 kb contains genes for degradation of benzene; etc. (Winstanley et al. 1987). The study of degradative pathways explained that genetic transfers by plasmid vehicles can overcome natural

Table 3.1 Plasmids reported in pseudomonads for degradation of aromatic hydrocarbons

Sr no.	Plasmids	Aromatic hydrocarbons degradation	References
1	pKF1	Chlorinated biphenyls	Shields et al. (1985)
2	TOL	Xylene, toluene, toluate	Yano et al. (2010)
3	NAH7	Naphthalene	Fernández et al. (2012)
4	pAC21	<i>p</i> -Chlorobiphenyls	Chatterjee and Chakrabarty (1983)
5	pAC25	3-Chlorobenzoate	Chatterjee and Chakrabarty (1983)
6	pAC27	3- and 4-Chlorobenzoate	Ghosal and You (1988)
8	pWW174	Benzene	Winstanley et al. (1987)

**Fig. 3.19** Catabolism of broad-spectrum aromatic xenobiotic compounds by pseudomonads into two central intermediates: catechol (a) and protocatechuate (b)

blockades in biochemical pathways which are preventing the degradation of complex (xenobiotic) aromatic compounds (Table 3.1).

Members of genus *Pseudomonas* can catabolize broad-spectrum aromatic xenobiotic compounds into two central intermediates that are catechol and protocatechuate (Fig. 3.19). In general, the aerobic degradation of aromatic hydrocarbons proceeds in two phases. First is their ring cleavage by a variety of ring modification reactions, and the second phase of degradation includes ring fission by either ortho-cleavage that occurs between the hydroxyl groups (intradiol cleavage) or meta-cleavage when it occurs adjacent to one of the hydroxyls (extradiol cleavage). The intermediates of these subsequent reactions ultimately converge to the central metabolism by formation of tricarboxylic acid cycle intermediates such as acetyl-CoA, succinyl-CoA, oxaloacetate, and pyruvate (Fig. 3.20).

3.3.3.1 Toluene Degradation

Toluene is naturally present in petroleum, coal, and gasoline. It is also produced by burning of organic materials and cigarettes and released in the environment as

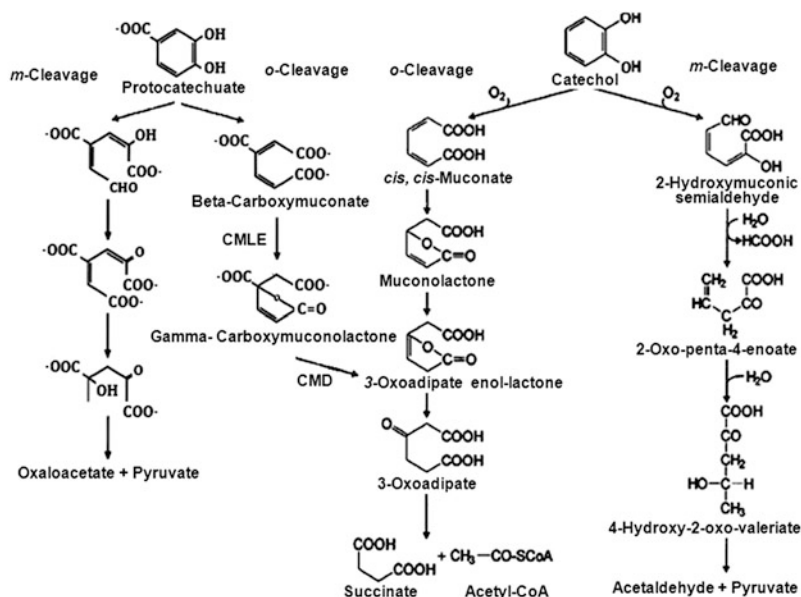


Fig. 3.20 Ortho- and meta-cleavage of catechol and protocatechuate: 3-carboxymuconate cycloisomerase (CMLE) and *cis*-muconate lactonizing enzyme (CMD)

solvent which is being used at industrial scale for production of different chemicals (Darrall et al. 1998; Koppmann et al. 1997; Sinninghe Damste et al. 1992; Heiden et al. 1999; Holzinger et al. 2000; Vrkocova et al. 2000). The highly reduced form of the toluene structure and stability of the benzene ring are the main factors affecting its utilization by microorganisms. The pseudomonads can use oxygen as a substrate for oxidizing and destabilizing the aromatic ring in toluene. These bacteria are using dioxygenase pathway where toluene dioxygenase (TDO) catalyzes the initial conversion of toluene to *cis*-dihydrodiol in *P. putida* F1 which gets converted to 3-methylcatechol by dehydrogenation. The enzyme catechol 2,3-dioxygenase (C23O) further oxidized 3-methylcatechol to 2-hydroxy-6-oxohepta-2,4-dienoate (HOD) which undergoes meta-cleavage. The pseudomonads (*P. mendocina* KR1) carried another monooxygenase-mediated toluene degradation pathway in which toluene is first oxidized at the para position by toluene 4-monooxygenase to form *p*-cresol, followed by oxidations of the methyl group to form 4-hydroxybenzoate (Klecka and Gibson 1981). A second ring hydroxylation generates protocatechuate, which undergoes ortho-cleavage to generate intermediates of TCA. Alternatively, pseudomonads may also use TOL pathway for oxidation of the methyl group of toluene by using transmissible plasmid TOL found in *P. putida* mt-2 (Williams and Murray 1974; Nakazawa 2002). The TOL plasmid-encoded pathway converts toluene to benzoate using three enzymes, xylene monooxygenase, benzyl alcohol dehydrogenase, and benzaldehyde dehydrogenase, which are ultimately converted to catechol (Fig. 3.21).

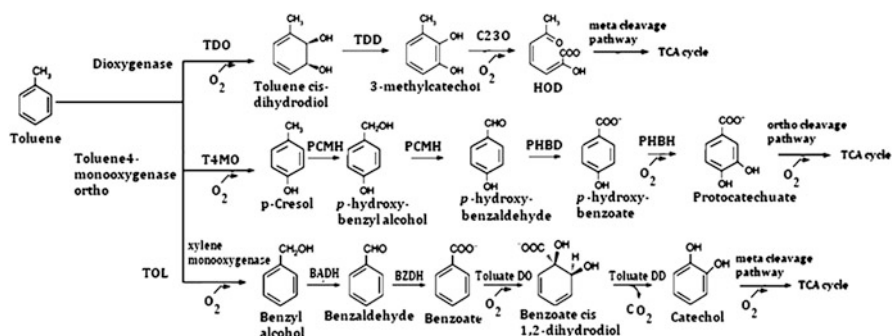


Fig. 3.21 Toluene degradation by pseudomonads: Dioxygenase-mediated toluene degradation pathway indicating intermediate: HOD, 2-hydroxy-6-oxohepta-2,4-dienoate; enzymes TDO, toluene 2,3-dioxygenase; TDD toluene cis-dihydrodiol dehydrogenase; C23O, catechol 2,3-dioxygenase; toluene 4-monoxygenase-mediated toluene degradation pathway in involves enzymes T4MO, toluene 4-monoxygenase; PCMH, *p*-cresol methylhydroxylase; PHBD, *p*-hydroxybenzaldehyde dehydrogenase; PHBH, *p*-hydroxybenzoate hydroxylase; TOL pathway enzymes: BADH, benzylalcohol dehydrogenase; BZDH, benzaldehyde dehydrogenase; toluate DO, toluate dioxygenase; toluate DD

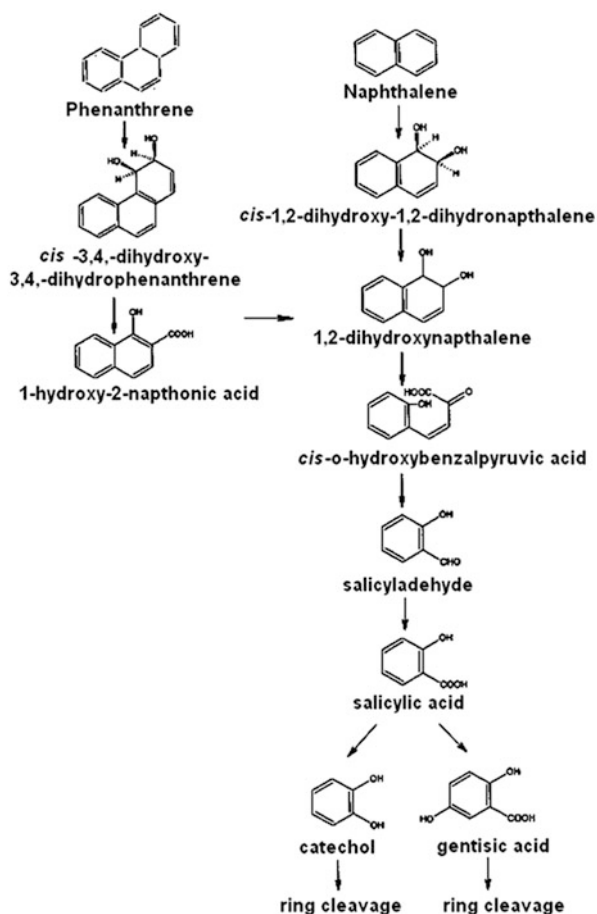
Thus, in the aerobic pathways, catechol and protocatechuate generally serve as central metabolites in toluene degradation which further leads to formation of tricarboxylic acid cycle intermediates supporting growth and energy requirements of pseudomonads.

3.3.3.2 Naphthalene and Phenanthrene Degradation

Naphthalene is an organic compound with formula $C_{10}H_8$ and consists of a fused pair of benzene rings. It is the main ingredient of traditional moth balls used as insect repellents. It is also used in synthesis of carbamate insecticides, surface-active agents, and resins. Most naphthalene is mainly derived from coal tar and heavy petroleum fractions during petroleum refining. Naphthalene is the simplest and water-soluble polycyclic aromatic hydrocarbon (PAH) that acts as a dye intermediate and synthetic tanning agent.

The enzymatic reactions for microbial degradation of naphthalene were first presented by Davies and Evans by soil pseudomonads (Davies and Evans 1964). Naphthalene dioxygenases from *Pseudomonas* sp. NCIB 9816 and *Pseudomonas putida* ATCC 17484 is a three protein components enzyme system responsible for oxidizing naphthalene by incorporating both atoms of molecular oxygen into the aromatic molecule to form *cis*-1,2-dihydroxy-1,2-dihydronaphthalene (Cerniglia 1984). The second step in oxidation of naphthalene is the conversion of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene to 1,2-dihydroxynaphthalene catalyzed by naphthalene (+)-*cis*-dihydrodiol dehydrogenase which requires NAD^+ as an electron acceptor. The next step leads to the enzymatic cleavage of 1,2-dihydroxynaphthalene to *cis*-2-hydroxybenzalpyruvate, which is then converted *via* dioxygenases to salicylate and pyruvate. Salicylate is oxidized by

Fig. 3.22 The proposed pathways for phenanthrene and naphthalene metabolism by pseudomonads



salicylate hydroxylase to catechol, which can undergo either *ortho*- or *meta*-cleavage. An alternative salicylate metabolism has been observed in *Pseudomonas testosteroni* which converted salicylate to gentisic acid (Fig. 3.22). Thus, catechol and gentisic acid serve as central metabolites in naphthalene degradation which undergoes ring cleavage to formation of tricarboxylic acid cycle intermediates (Houghton and Shanley 1994).

Phenanthrene a polycyclic aromatic hydrocarbon (PAH) is a composite of phenyl and anthracene composed of three fused benzene rings. It is a known irritant found in cigarette smoke. These PAHs are found naturally in the environment but can also be released on incomplete burning of coal, oil, gas, and garbage. Like most PAHs, phenanthrene is used to make dyes, plastics, pesticides, explosives, and drugs.

Phenanthrene is used as model substrates in studies on the environmental degradation of PAHs. This structure is found in carcinogenic PAHs such as benzo

[a]pyrene. In general bacteria from the genus *Pseudomonas* initially oxidize phenanthrene in the 1,2 and 3,4 positions to form *cis*-1,2-dihydroxy-1,2-dihydrophenanthrene or *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene. The ring cleavage product is further metabolized to 1-hydroxy-2-naphthoic acid, which is oxidatively decarboxylated to 1,2-dihydroxynaphthalene and then subjected to *meta*-cleavage to form salicylic acid (Gibson and Subramanian 1984). Salicylic acid can also be further degraded via the formation of either catechol or gentisic acid. Both catechol and gentisic acid undergo ring fission to form TCA cycle intermediates (Fig. 3.22).

3.3.3.3 Polychlorinated Biphenyl (PCB) Catabolism

Polychlorinated biphenyls (PCBs) were synthesized in 1880 by Schmidt and Schultz (1881) and were available commercially in 1929. The phenyl rings of PCBs may have 1–10 chlorines with 209 possible combinations. The mixtures of PCBs are being used as hydraulic fluids in electrical transformers and capacitors, as lubricating and cutting oils and as additives in plastics, paints, printing inks, adhesives, and sealants. The physicochemical properties of PCBs vary widely and depend on the number and positions of chlorine atoms in the biphenyl rings. In the environment, PCBs cycle between water, soil, and air, particularly concentrated in surface soils, river, and estuarine sediments due to their strong affinity to organic materials (Loganathan and Kannan 1994; Weber et al. 2008). Generally, water solubility and biodegradability of PCBs decrease with increasing number of chlorine atoms which makes certain PCBs highly persistent in the environment causing chronic toxicity to wildlife and humans. Their accumulation in the environment adapts the microbial flora of the site to survive in presence of PCBs and utilize these compounds as carbon and energy source (Hardy 2002; Huang et al. 2004; Wang et al. 2003). The microorganisms tend to breakdown the less chlorinated congeners of PCBs faster than the highly chlorinated ones (Bokvajova et al. 1994). These compounds can be degraded both aerobically and anaerobically. The anaerobes remove the chlorines without cleaving the biphenyl rings (Tiedje et al. 1993). On the other hand, aerobic microbes oxidatively break the aromatic rings of PCBs and substantially decrease the toxicity (Furukawa 2000).

Pseudomonads have been isolated and characterized for their ability to aerobically catabolize PCBs. *Pseudomonas* sp. usually degrades PCBs through 3,4-dioxygenation and 2,3-dioxygenation which allow these strains to metabolize a wide range of PCB congener.

The *Pseudomonas* sp. strain DJ-12 can degrade 4-chlorobiphenyl (4-CBP) and utilize it as a carbon and energy source. The *meta*-cleavage of 4-CBP involves four different enzymes: 4-chlorobiphenyl dioxygenase (PcbA), dihydrodiol dehydrogenase (PcbB), 2,3-dihydroxybiphenyl-1,2-dioxygenase (PcbC), and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (PcbD). The process catalyzed 4-CBP to 2,3-dihydroxybiphenyl (2,3-DHBP) and subsequently to 4-chlorobenzoate (4-CBA). The 4-CBA then undergoes hydrolytic dechlorination to 4-hydroxybenzoate (4-HBA) with the help of a set of three enzymes 4-CBA-CoA ligase (FcbA), 4-CBA-CoA dechlorinase (FcbB), and 4-HBA-CoA thioesterase (FcbC). The *Pseudomonas* strain further hydroxylated 4-HBA to protocatechuate

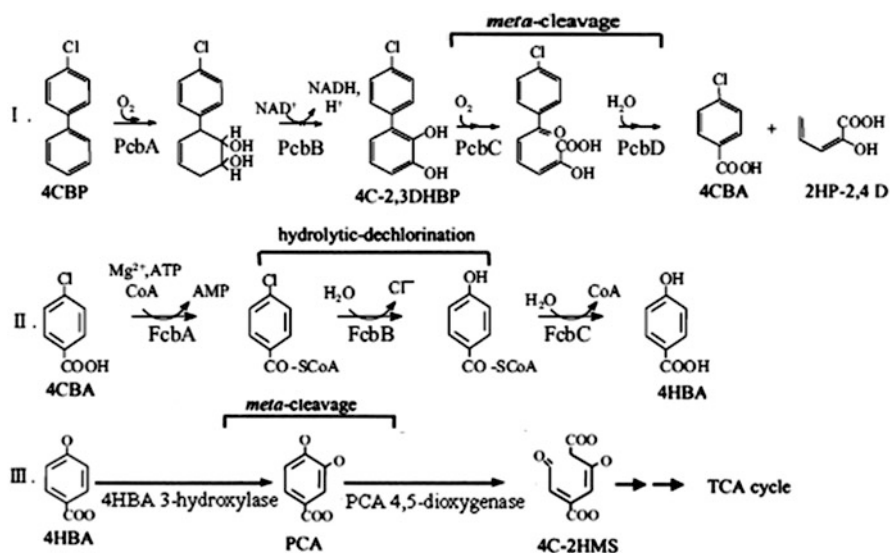


Fig. 3.23 The polychlorinated biphenyl (PCB) catabolic pathway by *Pseudomonas* sp.: involving three major steps: (I) dioxygenated degradation of 4-CBP to produce 4-CBA via *meta*-cleavage, (II) hydrolytic dechlorination of 4-CBA to 4-HBA, and (III) hydroxylation of 4-HBA to PCA which undergoes *meta*-cleavage to produce 4-C-2HMS. The enzymes involved in the first two steps are 4-chlorobiphenyl dioxygenase (PcbA), dihydrodiol dehydrogenase (PcbB), 2,3-dihydroxybiphenyl 1,2-dioxygenase (PcbC), 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolyase (PcbD), 4-CBA-CoA ligase (FcbA), 4-CBA-CoA dechlorinase (FcbB), and 4-HBA-CoA thioesterase (FcbC)

(PCA) by using 4-HBA-3-hydroxylase, and subsequently PCA-4,5-dioxygenase cleaves PCA at meta site to produce a linear structured compound 4-carboxy-2-hydroxymuconic semialdehyde (4C-2HMS) which is readily utilized as carbon and energy source via the TCA cycle (Fig. 3.23).

3.4 Conclusion

The microorganisms belonging to the genus *Pseudomonas* are ubiquitous and can live in both inanimate and human environments due to their surprising nutritional versatility. This versatility is due to the presence of a large number of genes that are expressed selectively in diverse environmental conditions to produce different sets of enzymes which allow pseudomonads to utilize various organic substances as nutrients and make them one of the most abundant organisms on earth. Pseudomonads can respire aerobically as its preferred metabolism but can also grow anaerobically using different inorganic electron acceptors. *P. aeruginosa*, a very successful inhabitant of soil, sewage, and wastewater, is capable of breaking down diverse range of aromatic hydrocarbons through its peripheral metabolic pathways. These aromatic xenobiotic compounds are broken down to two central

intermediates, catechol and protocatechuate, which converge to central metabolic pathway (EMP, ED, PPP, or citrate cycle) to make building blocks and energy for the cells. Moreover, pseudomonads are also reported to be major degraders of low water-soluble linear, cyclic, and branched alkanes spilled during refining, transport, and storage of petroleum products to alcohols, aldehydes, and organic acids. This suggests the future uses of pseudomonads for environmental detoxification of synthetic chemicals and formation of economically useful products from environmental waste.

The pseudomonads support breakdown of available amino acids in their surroundings via different peripheral pathways and convert to pyruvate, acetyl-CoA, oxaloacetate, fumarate, succinyl-CoA, and oxoglutarate. These complex enzymatic systems of pseudomonads allow utilization of amino acids for synthesis of TCA intermediates, fatty acids, NADH, and ATP which are essential for growth and survival of bacteria in diverse environmental conditions. *Pseudomonas aeruginosa* can switch from nonpathogenic non-mucoid to pathogenic mucoid form with synthesis of alginate by the process of gluconeogenesis which converts oxaloacetate into fructose 6-phosphate, a precursor for alginate production that makes it an opportunistic pathogen in patients with cystic fibrosis. Thus, this organism comprises both planktonic growth and alginate-associated biofilm formation. The large genome and ability to express a certain set of enzymes in the presence of a particular substrate or a set of substrates help *Pseudomonas* sp. in successful adaptation to multiple niches with extraordinary physiological capabilities which reflect the broad metabolic capacity of the organisms of this genus.

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Rachhpal S. Kahlon

Contents

4.1	Introduction	128
4.2	Genome Structure and Organization	132
4.2.1	Size	134
4.2.2	Genome Alignment	134
4.2.3	Core Genome	138
4.2.4	Accessory Genome	141
4.3	Comparative Functional Genomics	151
4.3.1	Metabolism, Transport and Regulation	151
4.3.2	Central Metabolic Pathway	157
4.3.3	Peripheral Metabolism and Bioremediation	158
4.3.4	Regulation and Signal Transduction	162
4.3.5	Comparative Pathogenicity	163
4.3.6	<i>Pseudomonas fluorescens</i> : Commensal and Plant Growth Promoter	171
4.3.7	<i>Pseudomonas stutzeri</i>	177
4.4	Summary	178
	References	179

Abstract

The genus *Pseudomonas* represents over 200 species of subclass γ -proteobacteria isolated from varied habitat and metabolic niches associated with array of activities such as pathogenicity towards human beings, animals, plants and insects and environmental bacteria with vast metabolic potential to degrade saturated hydrocarbons and a variety of manmade molecules used as pesticides; still others have considerable economic potential as biocontrol agents and production of commercially important metabolites such as biosurfactants, bioplastics, enzymes, etc. The diversity of the genus is reflected in its genome

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comprising of a single large circular chromosome with interspersed variable regions showing high degree of plasticity. The genome size varies between the smallest of *P. stutzeri*, 4,567,418 bp (4209 genes), and the largest of *P. bauzanensis* strain W13Z2, a halotolerant polycyclic aromatic hydrocarbon-degrading bacterium having 8.6 Mb (8170 genes), and *P. aeruginosa* PAO1 and *P. putida* KT2440 with 6,264,404 bp (5688 genes) and 6,181,863 bp (5516 genes), respectively, fall in between. The comparison of *P. putida* KT2440 and *P. aeruginosa* PAO1 shows that 85 % of the CDSs are homologous. The diversity of the genus *Pseudomonas* is also evident from the large pan-genome estimated to be 25,907 genes. The core genome of *P. aeruginosa* accounts for nearly 90 % of the total genome compared to 45–52 % in *P. fluorescens* strains, which means that large chunk of the DNA in the latter is acquired horizontally, thereby projecting greater diversity. Further expansion of the databases will provide a way for in silico modelling and prediction of biotechnological applications.

4.1 Introduction

Members of the genus *Pseudomonas* are diverse with respect to their physiology, metabolism and ecological habitat and form a large group under γ -subclass of proteobacteria. The striking feature is their ability to utilize a variety of compounds as sources of carbon and energy and production of an array of secondary metabolites. As a consequence to their vast adaptability, they inhabit soil as saprophytes or associate with plants as commensals in the rhizosphere and phyllosphere or as endophytes in plant tissues and are free living in aquatic environment and pathogens of human beings, animals, insects and plants. Presently, the genus *Pseudomonas* comprises of more than 200 species which have been isolated and identified from different ecological niches and sources. Because of their metabolic diversity and versatility, they play an important role in decomposition of organic matter, degradation of xenobiotic compounds and recycling of carbon, nitrogen and phosphorus in nature as they can solubilize the bound insoluble phosphorus (Stanier et al. 1966; Palleroni et al. 1973; Mulet et al. 2012). For last over three decades *Pseudomonas* have been subject of keen interest both in basic and applied research as they form an important group from the point of view of public health, agriculture, environment and bioremediation and are emerging as a model organism for exploitation for biotechnological applications. One strain, the soil bacterium *P. putida* KT2440, a TOL⁻ variant of *P. putida* mt-2, isolated from garden soil has been certified as “generally regarded as safe” (GRAS) for cloning and expression of foreign genes by the Recombinant DNA Advisory Committee of National Institute of Health, USA. This is the first Gram-negative soil bacterium to be “GRAS” certified.

Pseudomonas aeruginosa, an opportunistic pathogen of human beings and animals, is the type species of the genus *Pseudomonas* and is considered an “epitome” of opportunistic pathogenesis as it almost never infects the uncompromised healthy tissue; however, it can infect any tissue provided the tissue has some type of compromised defence mechanism. This can colonize a broad spectrum of habitat with the ability to exploit different nutritional sources and has high potential for adaptation to changing environment (Jensen et al. 2004). Besides their ability to subsist on different carbon and energy sources, they can utilize NO_3^- as terminal electron acceptor in the absence of oxygen, have minimum nutritional requirements and grow even at 42 °C. They produce a polysaccharide, alginate, and develop biofilms which help it to protect against host phagocytosis and multiply in the tissue. *Pseudomonas aeruginosa* strains are highly resistant to antimicrobials and can even grow in certain hospital disinfectants and solutions. As a result of such properties, it has been traced to nosocomial infections and can colonize various hospital devices such as catheters, bronchoscopes, etc. *Pseudomonas aeruginosa* is a source of septicemia in burn injury patients, urinary tract infections in catheterized patients and pneumonia in patients on respirators. It is a predominant cause of morbidity and mortality in patients with cystic fibrosis of lungs. Besides disrupting the host cell by combination of type III secretion effector proteins, *P. aeruginosa* produce a host of virulence factors including enzymes to degrade extracellular matrix, adhesins and exotoxin that inhibits host RNA translation and flagella synthesis. *Pseudomonas aeruginosa* isolates show considerable variability, for example, strain PA14, an ExoU-positive (a potent cytotoxin with phospholipase A activity) clinical isolate, displays much higher virulence than PA01; LESB58, a so-called “Liverpool Epidemic Strain” (LES), shows high transmissibility among CF patients (McCallum et al. 2002) and strain PA7 is a clinical isolate from Argentina with a notably unusual resistance to antimicrobials (Roy et al. 2010; Klockgether et al. 2010).

Pseudomonas putida, another important and well-documented species, are fast-growing bacteria that are frequently isolated from temperate soils and water particularly niches contaminated with organic chemicals and pollutants. They are nutritionally very versatile and play an important role in recycling of organic wastes in aerobic and microaerophilic environment and can utilize more than about 80 organic compounds as sources of carbon and energy (Clarke 1982; Timmis 2002). They have great potential in bioremediation of environments polluted with petroleum hydrocarbons, organic chemical solvents, polycyclic and heterocyclic compounds, etc. Degradation of a number of these compounds such as camphor, salicylic acid, benzene, toluene, xylene, naphthalene, benzoic acid and their chlorinated derivatives, biphenyls, alkaloid nicotine, etc. by *P. putida* is mediated by pathways encoded on plasmids (Chakrabarty 1976; Thacker et al. 1978; Ghosal et al. 1985; Saini and Kahlon 1998). Saprophytic *Pseudomonas putida* KT2440, the best studied strains of *P. putida*, is a plasmid-free derivative of toluene-degrading bacterium originally designated as *P. arvilla* strain mt-2 (Kojima et al. 1967) and subsequently reclassified as *P. putida* mt-2 (Williams and Murray 1974). This was isolated from a garden soil in Japan for its ability to utilize 3-methylbenzoate

(Nakazama 2002). An array of genetic tools based on mini-transposons have been developed for its analysis and manipulation and as a host for cloning genes from other soil organisms as well as for potential biotechnological applications, particularly for production of bioplastics (de Lorenzo and Timmis 1994). Strain KT2440 is being exploited for the development of commercial applications and designing novel catabolic pathways, production of vaccines (Rojo et al. 1987; Erb et al. 1997), production of intermediates for biosynthesis, biocatalysts and desulphurization of fossil fuels (Galan et al. 2000). KT2440 also colonize the rhizosphere of a number of field and garden plants and may facilitate the development of biopesticides and plant growth promoters (Espinosa-Urgel et al. 2002). *Pseudomonas putida* possess a number of enzymes for the degradation of aromatic compounds which are derivatives of lignin; the products of these metabolic reactions enter central metabolic pathways. Oxygenases and oxidoreductases play a key role in the transformation of recalcitrant compounds (Resnick and Gibson 1996; de Lorenzo et al. 2013).

Pseudomonas fluorescens have commensal relationship with plants; they draw their nutrients from the plant surface and root exudates and survive the environmental stresses by occupying protected sites. As commensals these have profound effect on plant's health and growth by suppressing pests, enhancing nutrient availability, altering physiological processes or degrading and eliminating environmental pollutants. They produce a variety of metabolites, including antibiotics that suppress fungal phytopathogens and promote plant growth. Some antibiotic-producing strains of *P. fluorescens* are used as biocontrol agents and have immense effect on agricultural productivity while some others produce growth hormones such as IAA and directly promote growth (Kraus and Loper 1992; Paulsen et al. 2005).

Pseudomonas protegens Pf5 (earlier *P. fluorescens* Pf5) is a rhizosphere commensal and is being commercially exploited as biological control agent because it produces an array of antibiotics and other secondary metabolites that suppress soil-borne plant pathogens (Pfender et al. 1993; Haas and Keel 2003) and has been extensively studied. The antibiotics produced by *P. protegens* Pf5 include pyrrolnitrin, pyoluteorin and 2,4-diaethylphloroglucinol (Howell and Stipanovic 1980; Nowak-Thompson et al. 1994). Besides this they also produce hydrogen cyanide, a metabolic inhibitor and siderophores which help in suppression of pathogens in rhizosphere through iron competition (Loper and Buyer 1991). *Pseudomonas fluorescens* P1CF7 is a native endophytic colonizer of olive (*Olea europaea* L) roots and antagonistic to fungal phytopathogen *Verticillium dahliae* Kleb, which causes *Verticillium* wilts in large number of plant species (Martinez-Garcia et al. 2015). The strain has been demonstrated as an effective biocontrol agent against *Verticillium* wilt of olive. Like *P. putida*, *Pseudomonas fluorescens* is diverse in catabolic capabilities and produces several extracellular hydrolytic enzymes such as chitinase, protease and lipases involved in the degradation of specific polymers. They also have capabilities for metabolism of plant-based sugars and more complex compounds such as long-chain hydrocarbons, aromatic compounds and heterocyclic compounds.

Pseudomonas entomophila was originally isolated from fruit fly and later found to be pathogenic to *Drosophila* (Vodovar et al. 2005) and is currently viewed as potential biocontrol agent for insect pests and saves the environment from toxic chemical pesticides. *Pseudomonas putida* is closely related to *P. entomophila* with 70 % of *P. entomophila* genes present in *P. putida* genome; 96 % of these are in synteny. They produce a number of degradative enzymes (lipases, proteases), toxins and secondary metabolites as virulence factors. The entotoxin produced by *P. entomophila* has a strong haemolytic activity due to cyclic lipopeptide-like structure. Upon ingestion, the systematic immune response system is activated both in *D. melanogaster* larvae and adult and infection causes disruption of the gut epithelium. *Pseudomonas entomophila* is able to kill insects from three other orders and as such it is a promising biocontrol agent against insect pests.

Pseudomonas syringae is a complex species comprising of several pathovars and these pathovars are specific for the host plant and are designated on the basis of the plant from which first isolated. The *P. syringae* strains are assigned to one of the more than 50 pathovars (pv), e.g. *P. syringae* pv. tomato DC3000 infects *Solanum lycopersicum* L. tomato as well as *Arabidopsis thaliana* as a model system (Dye et al. 1980); however, *P. syringae* pv. tomato T1 infects only tomato plants. The ultimate outcome of the plant pathogen interaction is the result of the interplay of multiple defence pathways and pathogen gene products. *Pseudomonas syringae* also produce a number of virulence factors such as phytotoxins, siderophores, adhesins, extracellular polysaccharides, pectolytic enzymes and other effector molecules for inactivation of antimicrobials during the host pathogenesis and injure cells of both host and nonhost plants. They also possess T3SS secretion system analogous to *P. aeruginosa* for injection of multiple effector proteins into the plant cell and interact with target proteins and suppress the host defence mechanism or manipulate hormone and/or elicit cell death. Epiphytic population of *P. syringae* may also carry the ice nucleation protein to catalyse the formation of ice nucleation at temperatures as high as minus 2 °C, while normally frost damage occurs at –4 to –12 °C. Freezing causes damage to the tissue and release of nutrients which help the bacteria to multiply and cause infection.

Pseudomonas stutzeri inhabit soil and colonizes plant root epiphytically and endophytically (Laluat et al. 2006). They are saprophytic and nonfluorescent as no fluorescent pigments are produced, which differentiates *P. stutzeri* from fluorescent pseudomonads. They are saprophytic and inhabit soil, water and marine waters and lack genes related to virulence including those for T3SS and T4SS (Yan et al. 2008). They show extensive metabolic functions and derive carbon and energy from sugars, starch, amino acids, acetate and pyruvate. In the absence of oxygen NO₃ can be used as terminal electron acceptor and carry out denitrification. In contrast some strains are diazotrophic and fix atmospheric nitrogen in a manner similar to *Azotobacter* spp. Thus they have potential as bioinoculants for nitrogen fixation and plant growth promotion. *Pseudomonas stutzeri* A1501 has been extensively studied and has a much smaller genome as compared to other *Pseudomonas* genomes. The vast spectrum of activities of *Pseudomonas* spp. is presented in Fig. 4.1.

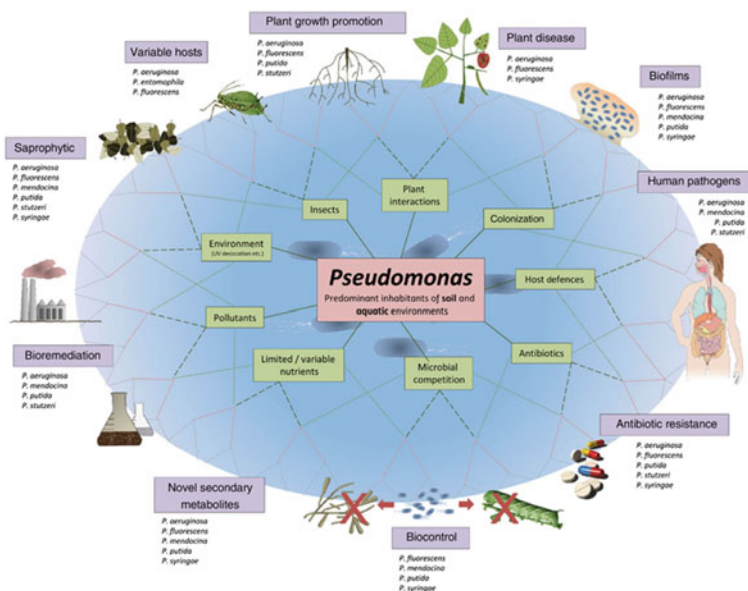


Fig. 4.1 The functional and environmental range of *Pseudomonas* spp. The *Pseudomonas* common ancestor has encountered a wide range of abiotic and biotic environments that has led to the evolution of a multitude of traits and lifestyles with significant overlap among species

The genetic material of *Pseudomonas* like other prokaryotes is represented by total DNA organized primarily into a single circular haploid chromosome and in addition there may be extrachromosomal DNA in the form of plasmids, transposons and phage genome. Up to 85–90 % of the DNA is non-repetitive and interspersed by integrative and conjugative elements (ICE) which may have been acquired by horizontal gene transfer. Genome sequencing and comparative functional genomics have been useful in providing basic structural and functional information. Many new genes have been identified and are being exploited for biotechnological applications. Functional genomic analysis of *Pseudomonas* represents various lifestyle issues and applications for these in health care and disease management, environment and bioremediation technologies, agriculture to improve soil health and promote plant growth, biocontrol of plant pathogens and pests and industrial biotechnology for production of small molecular compounds, enzymes and biopolymers of commercial interest.

4.2 Genome Structure and Organization

Pseudomonas is a large genus comprising of over 200 species known for their ubiquitous nature and are diverse with respect to their habitat and ecology. Genomes of about seventy strains of important species such as *P. aeruginosa*,

P. putida, *P. fluorescens*, *P. protegens*, *P. syringae*, *P. canabina*, *P. entomophila*, *P. mendocina*, *P. stutzeri*, etc. have been sequenced and compared (www.pseudomonas.com/genome; Stover et al. 2000; Nelson et al. 2002; Paulsen et al. 2005; Kung et al. 2010; Klockgether et al. 2011; Silby et al. 2011; Wu et al. 2011; Ozer et al. 2014). Members of the genus *Pseudomonas* have larger size of genome as compared to *E. coli* and related species. The genome of *Pseudomonas fluorescens* strains Pf5, now classified as *Pseudomonas protegens* Pf5, was considered the largest genome measuring 7,074,893 bp till recently, and now a larger genome consisting of 8.6 Mb with G+C ratio 61.8 % has been reported in halotolerant and polycyclic aromatic hydrocarbon-degrading, *Pseudomonas bauzanensis* strain W13Z2 (Wang et al. 2014). Given the vast spectrum of ecological, metabolic and biochemical characteristics of pseudomonads, it is apparent that the diversity of the species extends to the genome size also. Among the completed genome sequences, only 25–35 % of the genome of each strain comprises the core genome, i.e. the genes shared by all members of the genus *Pseudomonas*. New genes continue to be discovered within the species and strains, thus making it difficult to define a species in absolute terms of the genome sequence. In view of this a bacterial species, it can be described by its pan-genome which represents the sum total of (1) conserved core genome, i.e. genes present in all the strains, (2) the flexible/dispendable loci as “accessory genome” containing genes present in two or more strains and (3) the genes as “unique” which are specific to a single strain and are not shared with any other strain, of all the species/strains of the genus (Kiil et al. 2008; Seaton and Silby 2014).

The genome of all the strains sequenced to date is composed of one circular chromosome. Sequencing of multiple genomes within the species is important for better understanding of the diversity of the species. Besides, it has practical significance and the functional outcome of the gene may involve interplay of more than one gene. For example, it was only through multiple genome screening that all the four protein antigens could be identified to design a universal vaccine for group B streptococci (GBS) and the four proteins work additively as potent immunogens (Maione et al. 2005). Thus multistrain genome screening proved an effective approach to identify vaccine candidate and develop an effective universal vaccine for group B streptococci (*S. agalactiae*), group A streptococci (*S. pyogenes*) and pneumococci (*S. pneumoniae*). In the conventional technologies using single strain, the gene could be absent or have a limited expression and surface accessibility, thus rendering the particular fraction unidentifiable (Tettelin et al. 2005).

The genomes of *Pseudomonas* spp. show a highly mosaic structure, i.e. being composed of relatively stable core region being interspersed with variable regions accounting for plasticity of the genome, and are probably acquired through horizontal gene transfer. The genes that are unique to a strain are responsible for the distinctive feature of the strain, e.g. interaction with plant pathogens and target sites for biocontrol. The accessory genome represents phages, transposons, etc. derived from mobile genetic elements (Klockgether et al. 2011). Even considerable variability is observed between the strains of the same species. The comparison

of the genomes of four (Pf5, SBW25, Pf01 and WH6) strains of *P. fluorescens* shows tremendous diversity. Of the 5741–6009 predicted protein-coding genes identified in each genome, only 3115 are present in all the four strains. Thus, only 52–54 % genome of each of the strains accounts for the core genome. Furthermore, about one-third (1488–1833 genes) of the predicted proteome of each genome is unique to the specific strain.

4.2.1 Size

Members of the genus *Pseudomonas* are considered to possess large-sized genomes as compared to the model organism, *E. coli.*, and the sequenced genomes vary between 6 and 7 Mb except *P. stutzeri*. The genome of *P. aeruginosa* was reported as the largest when sequenced (Stover et al. 2000), but now few other strains have been reported to have still larger genome, e.g. *Bradyrhizobium japonicum* has a size of 9.1 Mb. Although the *Pseudomonas* species possess larger genome sizes as compared to pathogenic bacteria like *E. coli*, *Salmonella* species, *Mycobacterium* sp. etc but these measure well within the range of the genome sizes of environmental bacteria (Table 4.1).

Pseudomonas aeruginosa has the lowest AT content (33 %), while others vary between 38 and 42 %. The number of genes is around 5000 with variable number of rRNA operons. A fraction of global repeats include duplicated regions of chromosome such as multiple rRNA clusters; the average level of global repeats in bacterial genome is around 4 %. *Pseudomonas syringae* differs significantly from others and has higher number of repeats which may be attributed to higher number of transposable elements in their genome.

Pseudomonas aeruginosa has higher level of local repeats than other *Pseudomonas* genomes and this may be due to lower AT content. As the AT ratio of the base composition deviates from 50 %, it results in an increase in the chances of local repeats. As a consequent *P. syringae* with 42 % AT content have lower local repeats. All *Pseudomonas* genomes have higher fraction of direct repeats than inverted repeats (Achaz et al. 2002). *Pseudomonas* genomes have under-representation of purine stretches, while in general bacterial genomes, there tends to be over-representation. But alternating pyrimidine/purine stretches tend to be over-represented in *Pseudomonas* genomes. This bias is likely due to environmental factors (Ussery et al. 2002; Weinel et al. 2002)

4.2.2 Genome Alignment

Genome alignment of *P. aeruginosa*, *P. putida* and *P. syringae* showed that there were many rearrangements near replication origin but less sequence conservation near the replication terminal region was observed. Inversions around the origin of replication, particularly between two rRNA operons, are common in *Pseudomonas*

Table 4.1 Features of *Pseudomonas* genomes

Species	Strain	Size bp	A+T (%)	CDS (%)	Genes	rRNA	tRNA	Ref
<i>P. aeruginosa</i>	PA01	6264404	33.4	5576 (34.55)	5688 (35.25)	13 (0.09)	63 (0.4)	Stover et al. (2000)
	PA7	6588339	33.6	6286 (35.40)	6369 (35.86)	12 (0.07)	63 (0.36)	Roy et al. (2010)
	PA14	6537648	33.7	5892 (35.07)	5977 (35.57)	13 (0.08)	59 (0.36)	Lee et al. (2006)
	LESB58	6601757	33.7	5927 (34.48)	6028 (35.06)	13 (0.08)	67 (0.39)	Winstanley et al. (2009)
	M18	6327754	33.5	5684 (34.93)	5770 (35.56)	13 (0.08)	61 (0.38)	NCBI Genome Project
<i>P. putida</i>	KT2440	6181863	38.5	5350 (34.85)	5516 (35.91)	22 (0.15)	74 (0.74)	Nelson et al. (2002)
	NBRC14164	6156701	37.7	5449 (34.95)	5544 (35.65)	22 (0.15)	73 (0.73)	NCBI Genome Project
	DOT-T1E	6260702	38.6	5721 (34.97)	5803 (35.47)	7 (0.05)	58 (0.36)	NCBI Genome Project
	W619	5774330	38.6	5182 (34.77)	5309 (35.63)	22 (0.15)	75 (0.51)	NCBI Genome Project
	PIO-1	6438405	39.5	5722 (34.97)	5829 (35.63)	19 (0.12)	73 (0.45)	Silby et al. 2009
<i>P. fluorescens</i>	SBW25	6722539	39.5	5921 (34.83)	6106 (35.92)	15 (0.09)	66 (0.39)	Silby et al. (2009)
<i>P. protegens</i>	Pf-5	7074893	36.7	6108 (34.88)	6273 (35.61)	16 (0.1)	71 (0.41)	Paulsen et al. (2005)
	CHAO	6867980	36.6	6115 (34.92)	6199 (35.4)	15 (0.09)	68 (0.39)	NCBI Genome Project
<i>P. entomophila</i>	L48	5888780	35.8	5134 (34.55)	5275 (35.5)	22 (0.15)	78 (0.53)	NCBI Genome Project
<i>P. syringae</i>								
pv. tomato	DC3000	6397126	41.6	5481 (34.44)	5692 (35.77)	15 (0.1)	63 (0.4)	Buell et al. (2003)
	pDC3000A	73661	44.9	73 (33.67)	68 (36.14)	–	–	
	pDC3000B	67473	43.8	70 (33.34)	77 (36.67)	–	–	
pv. phaseolicola	1448A	5928787	42.0	4985 (34.29)	5228 (35.96)	16 (0.12)	64 (0.45)	Joardar et al. (2005)
	Large plasmid	131950	45.9	127 (32.16)	149 (37.73)	–	–	
	Small plasmid	51711	44.0	60 (36.59)	60 (36.59)	–	–	
pv. syringae	B728a	6093698	40.8	5089 (34.79)	5220 (35.68)	16 (0.11)	64 (0.44)	Feil et al. (2005)

(continued)

Table 4.1 (continued)

Species	Strain	Size bp	A+T (%)	CDS (%)	Genes	rRNA	tRNA	Ref
<i>P. stutzeri</i>	A1501	4567418	36.1	4127 (34.98)	4209 (35.67)	12 (0.11)	61 (0.52)	Yan et al. (2008)
	RCH2	4575057	37.5	4231 (34.65)	4368 (35.77)	12 (0.1)	59 (0.49)	NCBI Genome Project
	pPEST01	12763	46.1	16 (34.79)	16 (34.79)	–	–	
	pPEST02	9865	39.7	15 (33.34)	17 (37.78)	–	–	
	pPEST03	2804	38.2	3 (27.28)	4 (36.37)	–	–	
	DSM10701	4174118	36.8	3815 (34.77)	3888 (35.44)	12 (0.11)	61 (0.56)	NCBI Genome Project
<i>P. mendocina</i>	NK-01	5434353	37.5	4958 (34.96)	5035 (35.51)	12 (0.09)	65 (0.46)	NCBI Genome Project
<i>P. denitrificans</i>	ATCC13867	5696307	34.8	5056 (34.68)	5135 (35.22)	16 (0.11)	63 (0.44)	NCBI Genome Project

Ref. www.betapseudomonas.com; Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, Hancock RE, Brinkman FS (2011). *Pseudomonas* Genome database : Improved comparative analysis and population genomics capability for *Pseudomonas* genome. *Nucleic Acids Res.* 2011 Jan, 39 (Data issue): D596-600. Pubmed:20929876 NCBI Genome Project. <http://www.ncbi.nlm.nih.gov/genomes>

genome. Thus although many genes are conserved within the species, the relative location of within the chromosome is quite variable.

Gene comparison analysis of two *Pseudomonas aeruginosa* strains, two *P. fluorescens* strains and one *P. putida* and two *P. syringae* strains showed that *P. aeruginosa* strains have over 90 % proteins as common, *P. syringae* strains have 70 % and *P. fluorescens* strains have 60 % same proteins. This implies that *P. aeruginosa* have less genomic diversity than other species. Among the *Pseudomonas aeruginosa* strains only about 10% genes vary and the rest are homologous while the genomes of *Pseudomonas syringae* strains differ with about 45% homologs. Thus as with repeats, the genomes of *P. aeruginosa* and *P. syringae* stand out as distinct from other *Pseudomonas* genomes.

Comparison of whole genome between *P. putida* W619 and available sequence data on other pseudomonads revealed the presence of 3708 as shared coding sequences (CDS). Additional 684 CDS are shared between *P. putida* W619 and genomes of *P. putida* KT2440, GB1 and F1 strains (Fig. 4.2). Furthermore 82, 47 and 108 CDS are uniquely shared between W619 and strains KT2440, GB1 and F1, respectively. *Pseudomonas entomophila* L48 shares 110 additional genes with W619 and is considered closest to it. Notably 170 CDS of W619 have no hit to

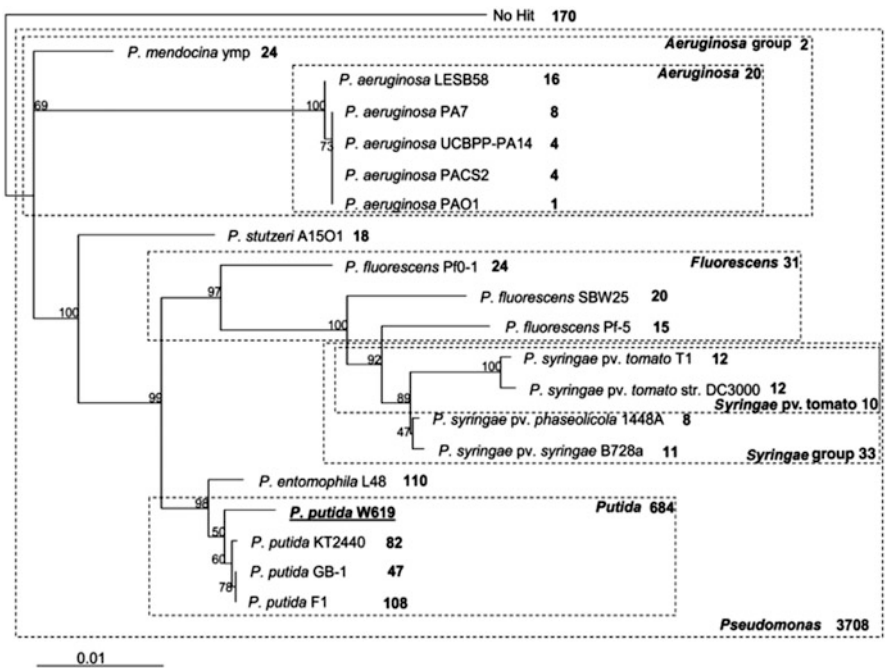


Fig. 4.2 Phylogenetic relationship between members of the genus *Pseudomonas* based on 16S rRNA gene comparison. The numbers at nodes represent the bootstrap values (1000 replicates), and the numbers in *bold* correspond to the number of coding sequences (CDS) preferentially shared by W619 and the corresponding organisms (with *E* value of 10^{-5}) (Huson et al. 2007)

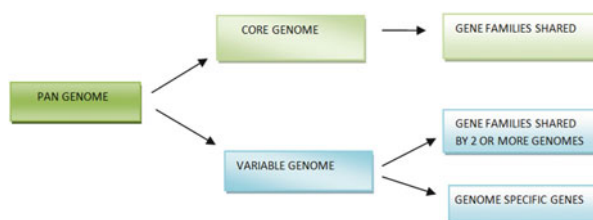
any of the sequenced *Pseudomonas* genome indicating that these genes have origin to some organisms outside *Pseudomonas*. The four strains of *P. putida* W619, F1, GB1 and KT2440 are predicted to code 5471 CDS with coding density of 89 %, 5300 CDS with coding density of 90 %, 5417 CDS with coding density of 90 % and 5420 CDS with a coding density of 86 %, respectively. All the four strains have a single circular chromosome. The chromosomal replication has a typical organization. The *oriC* is located between *rpmH* and the *dnaA* genes and contains conserved *dnaA*-binding boxes (TTATCCACA). *P. putida* W619 genome has considerable inverted alignments on both sides of the chromosomal replication origin (*oriC*) as compared to other *P. putida* strains. The region around the *oriC* seems to have undergone major rearrangement.

Pseudomonas as free-living soil bacteria adapt to a number of different environments through the regulation of expression of different sets of genes under different situations. Sigma factors, which initiate transcription through recognition of upstream genes, play an important role in this type of regulation. The number of σ -factors in an organism is the measure of its adaptability. Pathogens like *Mycoplasma* may have only one σ , while *P. aeruginosa* and *P. syringae* have 24 each and *Streptomyces coelicolor* has 65. The σ^{70} -related sigma factors are most abundant and diverse. *Pseudomonas syringae* is unique as it has least number, i.e. only 13 σ^{70} , while other species have at least 23 σ^{70} -related σ -factors. Less number of σ^{70} could mean that the loss of a single σ^{70} by mutation should result in non-availability of number of genes as they can't be transcribed. Thirteen extra-cytoplasmic (ECF) σ -factors were identified in genomes of *P. aeruginosa* and *P. syringae* with a similarity to *E. coli* FecI which is involved in iron acquisition (Roine et al. 1997).

4.2.3 Core Genome

The core genome of the species consists of those sequences that are conserved among the species, i.e. the genes that are present in all the strains of the species. This represents the minimum number of genes required for a bacterium to be considered as *Pseudomonas* genus. In general, the genes in the core genome contain the majority of housekeeping genes. On the other hand, the pan-genome is defined as the cumulative genetic information within the genome, i.e. any new gene characterized, which is not known to exist in the already sequenced genomes,

Fig. 4.3 Pan-/core/variable (accessory)/specific (unique) genome



will be added to the pan-genome (Fig. 4.3). As the new sequences of the genomes are released and the core gene component is refined, the core set of genes will reduce in size (Mathee et al. 2008; van Tonder et al. 2014). The core genome of *P. aeruginosa* containing 5.84 Mb represents 89.7 % (range 86.4–93.3 %) of the total genome. This has G+C content of 67.0 % as compared to 66.1–66.6 % of the complete genome. Thus core genome contains 5316 as predicted genes accounting for 90 % out of 5892 total coding sequences in PA14 and 5570, i.e. 95 % out of the total coding sequences of PA01. Twelve reference strains of *P. aeruginosa* (11 isolated from clinical samples and one, M18 from environment) were used to calculate the size of the core genome. Thus core genome represents nearly 90 % of the total genome of *P. aeruginosa* and is highly conserved. The genome size of *P. aeruginosa* strains falls in the range of 6.26–6.76 Mb with the average size of 6.52 Mb. Two distinct clusters of core genome size estimates were apparent depending upon which reference strains were included in the analysis. A cluster of smaller core genome size was obtained when PA7, a taxonomic outlier, was included in the analysis. Since it is relatively dissimilar to other PA strains, apparently the size of the core genome decreased when PA7 was used to define the core genome. Thus even the inclusion of a single outlier can affect the size of the core genome of *P. aeruginosa*. Ozer and his associates evaluated the phenomenon further by considering sequences shared by all strains, by at least 11 strains and by ten strains. This showed substantial increase in size of core genome. The differences were narrowed down to 436 kb of DNA segment conserved among the other eleven strains and were absent in *P. aeruginosa* PA7. These sequences encode several features characteristic of *P. aeruginosa* such as the exotoxin A gene, *tox A*. Thus they proposed that since an outlier genome significantly affects the definition of size of genome of a species, it may be excluded. They defined the size of core genome as those sequences that are present in at least eleven of the twelve ($\geq 90\%$) of the *P. aeruginosa* reference genome (Ozer et al. 2014).

Pseudomonas aeruginosa PA01 is an important opportunistic human pathogen and shows intrinsic resistance to antibiotics and disinfectants and are associated with nosocomial infections. This was the first strain of *Pseudomonas* spp. to be sequenced and its size is 6.3 Mb which was the largest of the sequenced bacterial genomes at that time; however larger genomes were reported later. The core genome as defined above represents sequence of $\geq 90\%$ of the reference genome. Therefore, the core genome contains 89.7 % (range 86.4–93.3 %) of the total genome. If the paralogs are excluded, the size of the core genome is reduced 5368 and 5520 genes for PA14 and PA01, respectively.

It is anticipated that as more completed genomes become available, the size of core genome will decrease and may plateau at 5.10 Mb or 78 % of *P. aeruginosa* genome. On the other hand as the cumulative genetic information within a bacterium's genome will become available, the additional genetic information to the pan-genome will enlarge the overall pool of genes resulting in increase in the size of the pan-genome of *P. aeruginosa* (Ozer et al. 2014). The genes or sequences that may be present in some strains and absent in other strains are referred to as “accessory genome.” Some of these may be present in two or more strains, but will

Table 4.2 Genes unique to each of the strains of *Pseudomonas* spp.

<i>Pseudomonas</i> isolate	No of the genes unique to the strain (Not shared with any of the strain)
<i>P. aeruginosa</i> 2192	187
<i>P. aeruginosa</i> C3719	45
<i>P. aeruginosa</i> LESB58	219
<i>P. aeruginosa</i> PA7	660
<i>P. aeruginosa</i> PACS2	29
<i>P. aeruginosa</i> PAO1	54
<i>P. aeruginosa</i> UCBPP-PA14	143
<i>P. protegens</i> Pf5	821
<i>P. fluorescens</i> Pf0-1	657
<i>P. fluorescens</i> SBW25	1195
<i>P. putida</i> F1	272
<i>P. putida</i> GB1	456
<i>P. putida</i> KT2440	422
<i>P. putida</i> W619	418
<i>P. syringae</i> pv. <i>oryzae</i> str 1_6	573
<i>P. syringae</i> pv. <i>phaseolicola</i> 1448A	263
<i>P. syringae</i> pv. <i>syringae</i> B728a	216
<i>P. syringae</i> pv. <i>tabaci</i> ATCC 11528	353
<i>P. syringae</i> pv. <i>tomato</i> DC3000	330
<i>P. syringae</i> pv. <i>tomato</i> T1	412

not be present universally in all the strains. In addition, there may be certain genes that may be present only in a particular strain and are not shared with any other strain within the species; such sequences are referred as “uniques” (Silby et al. 2011). The number genes unique to each strain of *Pseudomonas* are presented in Table 4.2.

Pseudomonas genome shows considerable flexibility and variations in the distribution of orthologous genes on the chromosome. Conservation of each protein-encoding gene along the chromosome is visualized in BLAST Atlas Data (Hallin et al. 2004). High degree of conservation has been observed around 817 kb, the region containing 36 genes involved in translation, ribosomal structure and biogenesis. Besides these proteins, the region also contains a ribosomal RNA operon and several tRNA genes. Thus it plays an important role in transcription and translation and as a consequence shows high degree of conservation. This region also shows specific structural features of “intrinsic curvature,” “stacking energy” and “position preference” due to the unusual base composition of rRNA genes and as such the unusual DNA structure of the genes and intergenic regions (Tatusov et al. 1997). This may be related to promoter activity to destack and unwinding of DNA for RNA polymerase binding and initiation of transcription. These special structures are indicators of strong promoters and high levels of expression of associated genes. The *Pseudomonas* genome shows a considerable flexibility as indicated by large

inversions and translocations such as those observed during long-term infections by *P. aeruginosa* (Ernst et al. 2003). An inversion of 1.7 Mb segment in *P. aeruginosa* PAO1 genome resulted in displacement of the replication terminus by 38.8 % on the circular chromosome.

Additional diversity has been observed due to changes such as duplication of a prophage region, deletions and SNPs, revealing microevolution in PAO1 and highlighting the plasticity of the genome (Klockgether et al. 2010). Similar inversion phenomenon attributed to plasmid and transposon was observed in *P. syringae* pv. tomato DC3000 (Landgraf et al. 2006). Resequencing of *Pseudomonas phaseolicola* Psy B728a genome identified 11 nucleotide substitutions representing mutations that might have arisen in the sample or errors in original sequence.

Extensive reciprocal recombination about the replication terminus in *P. fluorescens* has been reported (Silby et al. 2009). Some loci are inverted in one strain in relation to others and some other loci have been translocated with or without being inverted. The sequences that not susceptible to such rearrangements are found near the origin of replication and more rearrangements occurring near the replication terminus. This shows a high degree of stability around the origin of replication and more rearrangements taking place near the replication terminus. Repetitive sequences which may be involved in homologous recombination have been observed in *P. fluorescens* and related groups of *Pseudomonas* spp. such as *P. fluorescens* and *P. syringae* which are very tolerant to genetic rearrangement (Paulsen et al. 2005; Silby et al. 2009) as they have a high number of genes with no known function. However, the sequences into which RGP are inserted are highly conserved (Mathee et al. 2008).

Comparison of annotated genes in five strains of *P. aeruginosa* revealed extensive conservation of set of genes that are shared by all the five genomes. Among these 5021 genes are conserved across all the strains and share at least 70 % sequence identity. Among these, 90 % genes share at least 98 % sequence identity. Among the four strains of *P. fluorescens* Pf5, SBW25, Pf0-1 and WH6, only 3115 CDS out of 5741-6009 predicted protein-coding gene are present in all the four strains which represents 52–54 % of genome of each strain as core genome (Silby et al. 2009).

4.2.4 Accessory Genome

The core genome in *Pseudomonas aeruginosa* is highly conserved and constitutes nearly 90 % of total genome. In contrast, the accessory genome encompasses genes that are found in some and not in other strains. Thus the accessory genome is responsible for divergence in genome size. The accessory genome has an important role to play in *Pseudomonas* biology and contributes to metabolic diversity and confers specific phenotype to the strain that are advantageous under certain selective conditions, e.g. degradation of environmental pollutants such as hydrocarbons, chlorinated compounds and pesticides, fitness for disease, resistance to multiple classes of antibiotics, production of secondary metabolites, etc. The accessory

genome forms a mosaic structure by integration into the conserved extrachromosomal genetic elements like plasmids, prophages and insertion sequences in the core genome and are referred as regions of genomic plasticity (RGP). Accessory genome accounts for most intra- and inter-clonal genome diversity and is acquired through horizontal gene transfer from different sources and maybe even other genera and species. The integrated sequences into the conserved core genome appear as foreign blocks in core genome. The individual mosaics also show remarkable plasticity.

The acquisition of foreign DNA as well as different types of deletions/additions or mutations in single base or even gene inversions potentially affects the core and/or accessory genome and thus modifies the phenotype of the strain that differentiates from others (Klockgether et al. 2010). Besides their significance in ecology and adaptability of the host cell to different environments, the accessory genome has great medical relevance in *P. aeruginosa*. Accessory genome contributes to the persistence of *P. aeruginosa* in the host by production of virulence factors and also confers the ability to resist or inactivate antibiotics and drugs (Mesaros et al. 2007). Accessory genome is responsible for the rapid spread of such traits among the population. Due to the rapid spread of multiple drug resistance strains, *P. aeruginosa* has been declared as one of the “six top priority dangerous microbes” by the Infectious Diseases Society of America (Talbot et al. 2006).

4.2.4.1 Elements of Accessory Genome

Elements of accessory genomes are located in all sections of chromosome and are not concentrated in some regions. Nevertheless, the process of uptake of accessory DNA does not appear to be completely random but at some specific genomic loci that are prone to integration of mobile elements. On the basis of comparison of genomes of five strains PA01, PA14, 2192, C3719 and PACS2 of *P. aeruginosa*, Mathee et al. (2008) identified these regions of integration of mobile elements as “regions of genome plasticity” (RGP). Mathee and his colleagues looked for segments of DNA that are not conserved in all the five genomes and designated any region containing a block of four or more contiguous ORFs that is missing in at least one of the genomes as a RGP. For each of the RGP, they defined the DNA contained in accessory blocks and the ORF annotated within. The ORFs flanking the RGP regions are referred as “anchors” which describe the genomic site used for integration of foreign DNA. The genetic sequences in RGP larger than 10 kb are referred as genomic islands while shorter than 10 kb are called islets. Thus the islands are the horizontally acquired genetic element present in chromosome of some strains and absent in others. The RGPs form a large proportion of accessory genome. Once the foreign DNA gets integrated into host chromosome, it undergoes same pressures as the rest of the genome. The accessory gene pool has close association with mobile genetic elements and RGPs within the core genome act as hot spots for insertion of accessory genes. The tRNA genes in the core genome are frequently targeted for insertion of mobile genetic elements. On an average each sequenced *P. aeruginosa* chromosome carries 40 RGPs with insertions, strain PA7

has maximum of 53 RGP and tRNA gene is the integration site for 20 RGPs (Juhas et al. 2009).

The mobile genetic elements (MGE) such as phages, plasmids, transposons and genomic islands (GI) have been identified in all species of *Pseudomonas*, although they follow the same pattern of establishment, i.e. integration into the RGP in core genome they show variability with respect to their identity, location and function. Mobile genetic elements (MGE) have considerable effect on *Pseudomonas* genome but are less prominent in *P. entomophila* and *P. stutzeri* (Vodovar et al. 2006; Yan et al. 2008). Most species have a prophage-like elements which may be functional or only remnants, coding sequences (CDS) with similarity to transposase genes and plasmid-related sequences. The components forming accessory genome are grouped into following categories:

- (a) Integrative and conjugative elements (ICE)
- (b) Replacement islands
- (c) Prophages and phage-like elements
- (d) Transposons, insertion sequences and integrons

Since the accessory genomes are formed by combination of different functional modules, sometimes it is difficult to assign them to one category unambiguously and there may exist some overlapping.

(a) Integrative and Conjugative Elements (ICE)

Many genomic islands in *P. aeruginosa* are either integrative and conjugative elements (ICE) or derived from such elements. These are self-transmissible genetic elements that must integrate into an existing replicon for their replication. ICEs have properties of both of a plasmid, i.e. exist as extrachromosomal elements and transfer by self-mediated conjugation, and that of a prophage as their integration is mediated by phage integrase. The integration occurs at specific recombination sites, *attP* on the ICE and *attB* on the bacterial chromosome. Thus the genomic islands of *P. aeruginosa* are the category of ICE that may be divided into two subclasses, i.e. the one that are mobile and those that have lost their properties of mobility and become fixed due to degeneration of their phage or conjugative elements. Unlike plasmids most ICE don't replicate on their own but depend upon the chromosomal replication.

The mobilizable ICE of *P. aeruginosa* range from 81 to 108 kb and share a set of 72 ORF with >75 % sequence identity (Klockgether et al. 2004, 2007). These are highly conserved and mediate their excision, self-transfer to the new host and integration. A number of integrases are involved in the process (Sentchilo et al. 2003). On excision, the ICE circularizes to form an *attP* site and restore *attB* on *P. aeruginosa* chromosome. Conjugative transfer is then mediated by type IV secretion system (T4SS). The ICE-associated T4SS, referred as "genomic island (GI)-type T4SS," are widely distributed and are probably exclusive for genomic islands. The GI-type T4SS have been identified on genomic islands in *Salmonella enterica* serovar typhi, *Erwinia carotovora*, *Pseudomonas* spp., etc.

ICEs of *P. aeruginosa* fall in two large families: pKLC102-related ICEs and *clc*-like ICEs. The family pKLC102 includes pKLC102 itself along with GI4 (PAGI4), PAGI5, *P. aeruginosa* pathogenicity island 1 (PAPI-1) and PAPI-2. Each of these has evolved from pKLC102 originally identified in *P. aeruginosa*. The ICE pKLC102 is widely distributed, has a size of 104 kb and integrates into the chromosome at the *attB* site consisting of 15–20 bp located at the 3' end of two distinct tRNA^{lys} genes. The pKLC102-subtype islands (pKLC102, PAPI-1, PAGI-5) are endowed with *xerC/xerD*-like integrase gene and two copies of tRNA^{lys} gene in RGP7 and RGP41 can be used for insertion. In *P. aeruginosa* pKLC 102 appears to be an aberration of ICE as it shows high spontaneous mobilization frequency, occurs as 30 episomal copies per cell and has its own origin of replication (*oriV*). As such it may be appropriate to recognize it as a conjugative plasmid (Klockgether et al. 2007). However, the pKLC102 *oriV* has 16 highly conserved 57 bp direct repeats on one end and an AT rich region preceded by four palindromes on the other end (Wurdemann and Tummeler 2007). Outside the conserved backbone, pKLC102 contains a myriad of cargo genes, viz. genes coding for novel fatty acid synthases, the products of a putative chemotaxis operon, a cold adaptation protein, a polyketide synthase, a phage anti-repressor, four putative transcription regulators and a synthase for cyclic β -(1,2) glucan (Klockgether et al. 2004).

PAGI-4 is a pKLC102-like ICE that has become immobilized through a series of deletions. One border of PAGI-4 has a sequence identical to integrase-including segment of pKLC102 and integrates at the tRNA^{lys} gene inserts in RGP7. PAPI-1 and PAGI-5 are ICE that endow enhanced pathogenic characteristics on the host strain. PAPI-1 is 108 kb ICE identified in broad host range pathogenic strain PA14. PAPI-1 is located next to a tRNA^{lys} gene in the region around 5250 kb in the genome of PA14. This region shows only partial similarity to other distantly related *Pseudomonas*. PAPI-1 has a cluster of about 100 genes related to pathogenicity, some of which are homologous to known genes with virulence functions in other human and plant pathogens (He et al. 2004). PAPI-1 island potentially contributes to the evolution of variants with enhanced pathogenicity. Inter-strain transfer of a circular form of PAPI-1 is mediated by the PAPI-1 GI-type T4SS, which utilizes a self-encoded type IV pilus for conjugation. Synthesis of the pilus requires a pre-pilin peptidase encoded within the core genome of *P. aeruginosa*. Many of its cargo ORF promote pathogenicity and mutation in these results in reduced virulence. PAGI-5 has been identified in highly virulent strain of *P. aeruginosa* that causes pneumonia and is a 99 kb ICE similar to PAPI-1. PAGI-5 carries two regions of cargo ORF (NR-I and NR-II) that are absent in PAPI-1. The ORF of PAGI-5 are also associated with pathogenic functions. In addition, it also contains a cluster of genes with homology to the mercury ion-induced transcriptional regulator gene, a mercuric ion reductase gene and mercuric ion transport protein gene.

Another ICE PAPI-2 also has virulence properties. This island is 11 kb element with G+C of 56.4 % which is much lower than the overall GC ratio of chromosome of *P. aeruginosa*. PAPI-2 belongs to family of genomic island that carry genes coding for ExoU, a phospholipase effector protein secreted by *P. aeruginosa* type III secretion system which acts as virulence factor. Other PAPI-2-related islands

that carry gene coding for ExoU protein are *exoU* islands A, B and C. The PAPI-2 and ExoU islands form a related subset of ICEs within large pKLC102 family. Acquisition of these islands enhances virulence and contributes to pathogenicity or fitness (Battle et al. 2009; Jackson et al. 2011).

The Liverpool Epidemic Strain (LES) of *P. aeruginosa* is successful and aggressive transmissible strain associated with lung infections in CF patients. The genome of LES58 harbours all but two virulence genes. However, significant variations were noted with respect to duplication of pyoverdine-associated genes and divergence in gene complement and homology of type IV pili and phenazine biosynthesis genes. In strain LESB58, more than 75 genes were found on three novel islands. The 110 kb LESGI-3 showed a bipartite structure with 67 kb region identical to PAPI-2. One of these carries genes for biosynthesis of pyoluteorin, an anti-fungal agent found in *P. fluorescens*, while other two islands have genes encoding regulator, transporter, sensor and restriction modification (Winstanley et al. 2009). The major difference between genome of LESB58 and other *P. aeruginosa* strains was the presence of five GIs and six prophages, some of which were novel while others show similarity to elements in other sequenced strains. All the five GIs were common to LES isolates (with the lowest frequency observed being 86 % for LESGI-5, the only island unique to LES isolates). Only two of the five GIs identified within the LES strain showed similarity to any previously identified *P. aeruginosa* island, with the last 67 kb of the 110 kb LESGI-3 island showing similarity to PAPI-2, PAPI-3, PAPI-5 and PAPI-1, while LESGI-4 shared 46 % identity with PAPI-1 over its entire length. This is consistent with previous evidence that GI are a major source of novel genes for a genome (Winstanley et al. 2009). The LES genomic island (LESGI-3, 111 kb) and islands PAPI-2 (105 kb) and PAPI-3 (103 kb) belong to a family of *clc* like elements of *P. aeruginosa*. The *clc* element, first identified in *Pseudomonas knackmussii* strain B13, is 105 kb genomic island carrying genes for 3-chlorobenzoate metabolism. The cargo ORF besides carrying *clcRABDE* genes coding for enzymes for 3- and 4-chlorocatechol degradation also include functional operons for 2-aminophenol degradation, a putative aromatic compound transport gene and a dioxygenase gene. The *clc* elements are transmissible to genera classified under γ -proteobacteria (Gaillard et al. 2006, 2008; Springael et al. 2002). The site of integration, *attB* for *clc* elements, lies within the 3' end of two tandem tRNA^{GLY} genes on the chromosome and is mediated by a phage P-4 like integrase. Two other ICE of *P. aeruginosa*, viz. PAPI-2 and PAPI-3, also contain P-4 like integrase genes. The cargo ORF of these ICE along with LESGI-3 encode protein complexation with and transport of heavy metals and likewise cargo of PAPI-3 ORF codes for metabolic features, transport and resistance capabilities.

Nine GI have been identified in *P. protegens* Pf5, some of which encode secondary metabolites and six phage-like regions resembling phages of enteric bacteria (Mavrodi et al. 2009). These GI insert into hot spots such as between *mutS* and *cinA* genes where *P. protegens* has prophage-like elements. *Pseudomonas putida* GB1, *P. syringae* pv. tomato DC3000 and *P. entomophila* have various other genes. The *P. syringae* ICE island, PPHGI-1, carrying a T3SE undergoes excision

from the chromosome of *P. syringae* during growth in the resistant host and transfer to other recipient strains by conjugation/ transformation, thus leading to other genomic changes in the recipient.

(b) Replacement Islands

Virulence of *P. aeruginosa* is determined by its ability for cell to cell interaction, iron uptake, adhesion, mobility and production of virulence factors. This means that lipopolysaccharide (LPS) O antigen, pyoverdine, pili and flagella are critical determinants of *P. aeruginosa* fitness. The genes responsible for their synthesis and post-transcriptional modification are grouped together in gene clusters referred as “replacement islands” (Smith et al. 2005). Each of these gene clusters contains horizontally acquired components and is highly divergent between different strains. In contrast to other genomic islands, these genetic elements are present and occupy the same site in nearly all *P. aeruginosa* genomes. The multiple functions of these islands are to resist killing by phages or pyocins and to escape detection by host immune system.

The outermost part of LPS constitutes the O antigen, the polysaccharide of repeating sugar moieties. The structure of O antigen may vary with respect to structure and chain length and the variants are referred as serotypes. Twenty different O antigen serotypes of *P. aeruginosa* have been recognized. The genes encoding enzymes for O antigen biosynthesis are found in a single cluster that occupies the common genetic locus in all *P. aeruginosa* strains except for serotypes O15 and O17. The G+C ratio of these genes is lower than the chromosome of *P. aeruginosa* and signifies its acquisition by horizontal gene transfer (HGT).

Major siderophore produced by *P. aeruginosa* is a quinoline-derived chromophore, pyoverdine which gives a characteristic yellow green fluorescence, and has a peptide chain of variable length synthesized by non-ribosomal peptide synthetase (NRPS) (Meyer 2000). *Pseudomonas aeruginosa* strains produce three different types of siderophore I–III and thus grouped into three siderovars. Each strain produces a specific pyoverdine and pyoverdine receptor combination as the receptors are specific for pyoverdine. The genetic locus for pyoverdine contains genes for pyoverdine receptor, genes coding for non-ribosomal peptide synthase and the putative ABC transporter gene which is highly divergent among *P. aeruginosa* strains (Spencer et al. 2003). Diversity of pyoverdine receptors is attributed to recombination, positive selection and horizontal gene transfer (HGT). Although the loci have G+C ratio similar to *P. aeruginosa* chromosome but exhibit unusual codon and tetranucleotide usage. The type I and type II pyoverdine receptors are similar to receptors of *Azotobacter vinelandii* and *Agrobacterium tumefaciens*, respectively. The G+C content of these two soil bacteria is very similar to *P. aeruginosa* and this could be the reason for similarity of the genes responsible for pyoverdine receptors.

The type IV pili of *P. aeruginosa* are polymers of monomeric subunit of pilin and mediate adherence to the host cell surface and biofilm formation (Sriramulu et al. 2005). Five different groups of genes encode pilin and each group is unique in terms of sequence and length of pilin that it encodes.

The single polar flagellum which mediates motility and adherence is the potent stimulator of the innate immune response. Flagellum is a polymer of flagellin protein subunit and in *P. aeruginosa* flagellum is glycosylated. Flagellum is classified as a-type or b-type depending upon the variations in flagellin protein and its glycosylation. The flagellin glycosylation islands are thought to be horizontally acquired. Glycosylation also has a role in virulence of the *P. aeruginosa* strains (Arora et al. 2005). The b-type flagellin is conserved in sequence and glycosylation, while a type flagellin show greater diversity due to differential glycosylation pattern and presence of six *fliC* single nucleotide substitution (SNP) haplotypes (Verma et al. 2006). High degree of variability in the glycosylation cluster (RGP-9) has been reported. The RGP share 70 % sequence identity between homologs (Mathee et al. 2008). RGP-9, RGP-31, RGP-60 and RGP-73 carry gene clusters for flagellin glycosylation, O antigen biosynthesis, pilin biosynthesis and pyoverdine synthesis, respectively. The type of each replacement island was identified by comparative sequencing of the respective gene clusters in *P. aeruginosa* strains.

(c) Prophages and Phage-Like Elements

The temperate phages develop lysogenic relationship with host genome by undergoing site-specific recombination. Once integrated into the host genome, the bacteriophage is referred as prophage and behaves as an integral part of the host genome. The prophage may confer novel properties such as toxin production upon the host. As a result of exposure to certain DNA damaging stimuli, the prophage may undergo excision and in the process may lose some of its genes and may pick up the genes from the host chromosome and get packaged into the phage head. Such particles are referred as specialized transducing particles and can transfer the acquired trait to the new host.

Bacteriophages are highly abundant and there are at least 60 different temperate phages that infect *Pseudomonas aeruginosa* isolates. The *P. aeruginosa* phages are double-stranded DNA and tailed phages and on the basis of their tail morphology, they are divided into three groups: (1) *Siphoviridae* family of long, non-contractile tailed phages, which are LPS-specific double-stranded DNA phages and may mediate an alteration in LPS of bacteriophage, e.g. D₃ Kropinski (2000); (2) *Myoviridae*, the double-stranded DNA phages which contain *ctx* gene coding for pore-forming toxin, e.g. bacteriophage ϕ CTX (Nakayama et al. 1999); (3) *Podoviridae* family, filamentous phages, e.g. PT-6 and F116 that produce factors that digest the exopolysaccharide alginate.

Phages of *P. aeruginosa* are a reservoir for genetic diversity and the phages of *P. aeruginosa* are ubiquitous in nature and contribute to virulence and biofilm development. A study of 18 diverse *P. aeruginosa* phages revealed that 82 % of the predicted *P. aeruginosa* phage proteome is of unknown functions. The phage ϕ CTX a double-stranded Myoviridae family phage contains *ctx* gene coding for pore-forming toxin and enhances virulence (Baltch et al. 1994; O'Callaghan et al. 1996). D₃, a lysogenic bacteriophage, is a LPS-specific phage and brings about phage conversion as its insertion in PAO1 strain converts the serotypes O5 to O16. The strains lysogenized with D₃ display enhanced adherence to human buccal

epithelial cells. Bacteriophage F1Z15 which is similar D₃ is associated with increased resistance to phagocytosis. The filamentous phage, Pf4, mediates the appearance of small colony variants (SCV) that have enhanced antibiotic resistance (Webb et al. 2004). Bacteriophages DT-6 and F116 of *Podoviridae* family produce factors that digest exopolysaccharide, alginate, which is important in biofilm formation.

Many *P. aeruginosa* strains harbour two tandem phages derived from P₂ and λ phage that produce the R- and F-type pyocins, respectively. Pyocins are defective phages as they contain only phage tail genes and lack the genetic information for formation of head, replication and integration. The R- and F-type pyocins encode nuclease and protease-resistant rod-like particles resembling tails. They act by producing pores in the membranes of nonpyocinogenic strains. The R-type pyocins are active against *Neisseria gonorrhoeae*, *N. meningitidis*, *Haemophilus ducreyi* and *H. influenza* (Chang et al. 2005).

The prophages of *P. aeruginosa* often depict variations in their genomes and exhibit a mosaic structure and may share duplicated regions (Winstanley et al. 2009). The diversity of prophages is driven by genetic duplication, rearrangement and recombination and in turn the prophages contribute to genetic instability in the host strain during infection (Fothergill et al. 2010).

(d) Transposons, Insertion Sequences and Integrons

Multiple resistance to antibiotics was first reported in Japan in 1956 in *Shigella dysenteriae*, when it was considered that the resistance is mediated by extrachromosomal genetic elements, the plasmids. Later it was established that the transposable elements located within the plasmids were responsible for genes coding for resistance to antibiotics. In 1980s it was found that the genetic system responsible for the gathering of resistance determinants on transposons was “integrations” (Stokes and Hall 1989; Liebert et al. 1999). Insertion sequences are the transposable elements but are small in size and don’t carry any resistance markers. All transposable elements contain a gene or a group of genes encoding transposase or transposase complex.

The integrons are the genetic entities that capture exogenous gene cassettes and ensure their expression (Fig. 4.4). The integrons are composed of three essential components: (1) the *intI* gene, which encodes an integron integrase (*intI*) a member of tyrosine recombinase family and catalyses recombination between the host chromosome (*attC*) and incoming gene cassette (*attI*); (2) the integron that carries the primary recombination at site, *attI*; and (3) an integron-associated promoter, *P_c*, for expression of the core cassette (Collis and Hall 1995; Partridge et al. 2000; Recchia and Hall 1995).

Integrations acquire new genes as a part of gene cassettes, which are simple structures consisting of a single reading frame (ORF). Recombination between *attC* site (59-base element) and the *attI* mediated by integron integrase is reversible and the cassettes can be excised as free circular DNA elements (Rowe-Magnus et al. 1999). Insertion at *attI* site allows expression of an incoming cassette by the adjacent promoter, *P_c* (Collis and Hall 1995; Hocquet et al. 2012). Integrin system has two advantages as a means of genomic innovation: (1) New genetic material is

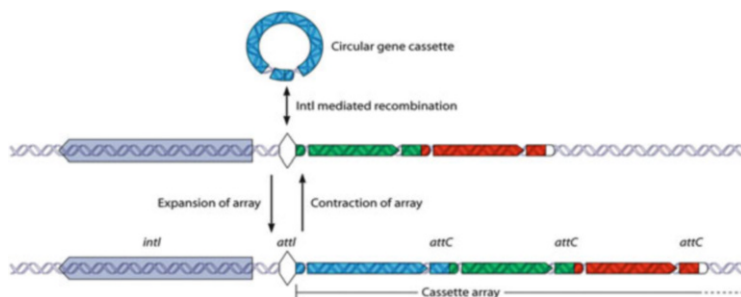


Fig. 4.4 Acquisition of cassette by recombination between *attC* of the circular cassette and the *attI* site of the integron. The cassette array can expand by repeated cassette acquisition and cassettes can also undergo excision as closed circles *attI* $\times$ *attC* or *attC* $\times$ *attC*

integrated at the specific site (*attI*) and thus does not disturb the existing genes. (2) The newly integrated gene is then expressed via the integron promoter (*P_c*) and is readily subjected to natural selection. Thus the newly generated variant will immediately express gene that might confer advantageous phenotypes (Bennett 2004).

The integrons carry antibiotic resistance gene cassettes and are common in *P. aeruginosa*. These are associated with Tn 402-derived transposons and are found in wide range of nonpathogenic bacteria and environmental bacteria. Examination of bacteria from soil, freshwater and biofilms suggests that 1–5 % cells carry a class 1 integron and they may go up to 30 % (Gillings et al. 2008; Riccio et al. 2005). They are the major factors in dissemination of antibiotic resistance and represent the classical example of natural selection, especially when applied to organisms with large population size, rapid growth and access to vast pool of genetic novelty. They have become abundant and are found in 40–70 % of Gram-negative pathogens as well as commensal flora of livestock and companion animals and even plant pathogens. The clinical integrons will continue to accumulate new gene cassettes encoding antibiotic resistance and other adaptive phenotypes. This will play role in genetic rearrangements with transposons, plasmids and other mobile elements (Kung et al. 2010).

Class 2 clinical integrons are associated with the Tn7 transposon, whose transposition is directed at specific attachment sites on chromosomes or plasmids. Metagenomic studies have detected functional class 2 integrase gene in agricultural habitats, associated with *Firmicutes*, *Bacteroides* and *Providencia stuartii* with unknown functions. This may be an environmental integron (Rodríguez-Minguela et al. 2009; Ramírez et al. 2010).

Integron-associated antibiotic resistance genes have been identified in several outbreaks of strains producing metallo- β -lactamase (MBL) as well as increased expression of extended spectrum of β -lactamases (ESLB) (Gibb et al. 2002; Kouda et al. 2009; Ramírez et al. 2010). The integrons generally carry MBL genes along with other antibiotic resistance determinants such as genes encoding aminoglycoside acetyltransferases, phosphotransferases and adenylyltransferases (Walsh et al. 2005). Due to their ability to capture and expression of multiple

resistant gene cassettes, integrons are major contributors for the development of multidrug-resistant strains of *P. aeruginosa*. The antibiotic resistance gene cassettes in the outbreak-related strains of *P. aeruginosa* have been traced back to environmental strains of bacteria. Environment appears to be an inexhaustible source of genetic diversity and constitutes a deep reservoir from which antibiotic resistance genes can be acquired and disseminated among *P. aeruginosa* (Stokes et al. 2001; Holmes et al. 2003).

The resistance genes and integrons emanating from human-dominated ecosystems are regarded as “xenogenetic pollutants” because DNA elements have been assembled under continuous selection exerted by human antibiotic use (Pruden et al. 2006; Storteboom et al. 2010). Unlike conventional pollutants, integrons and resistance genes can replicate and therefore have properties of both pollutants and invasive species (Tenaillon et al. 2004; Gillings and Stokes 2012; Gillings 2013). It is estimated that in the United Kingdom nearly 10^{19} bacteria harbouring class 1 integrons are released annually via sewage sludge. Resistance genes and integrons are present in floc sludge and even released into reclaimed water which may be disposed into rivers and may find way to ocean. Resistance genes and integrons are also disseminated through hospital waste and waste waters from tanneries. Even the use of animal waste as manure introduces genes in agricultural soils (Byrne-Bailey et al. 2011; Cheng et al. 2013; Chen and Zhang 2013).

4.2.4.2 Evolution of Accessory Genome

Horizontal gene transfer plays a major role in the structure of accessory genome of *Pseudomonas* spp. Besides HGT even strain-specific mutations causing deletions and additions, genetic rearrangements and duplications followed by clonal expansion can transform genomic regions that were found in all strains as unique accessory regions. Large chromosomal deletions resulting in loss of pyoverdine synthesis and uptake in *P. aeruginosa* strains isolated from CF patients resulted in selective reduction in core genome and became part of the accessory genome organization. Thus, these ongoing processes along with HGT continually modify the content of accessory genome. Only two HGT mechanisms, i.e. transduction and conjugation, are well documented in *P. aeruginosa*, though natural transformation process involving type IV pilus is not ruled out (Hupkova et al. 1994). Treatment with agents such as mitomycin C, use of antibiotics, UV light and physical stress also promotes mobilization of prophage in *P. aeruginosa*. Once mobilized, the accessory elements may be transferred to new host but the efficiency of the process depends upon number of factors such as host background and its relatedness to the previous host.

Comparative analysis show that the highly host adapted pathogens and symbionts undergo genome reduction and the environmental organisms continually expand their genomic repertoires. *Pseudomonas aeruginosa* has been able to customize its genome by using genetic mechanisms that facilitate the movement and alteration of genetic material in a way to fit its genome to the needs for survival in virtually any environment. One of the striking examples of genome expansion is the acquisition by PA2192 of a large cluster of 95 genes involved in abietane diterpenoid metabolism (Dit Island comprising of PA2G-1975 to PA2G-2069).

This gene cluster is added en bloc to its genomic repertoire allowing this particular strain to grow in environments rich in abietane diterpenoid resins that are produced by tree plants as defence molecules. *Pseudomonas abietaniphilia* and *Burkholderia xenovorans* can use abietane diterpenoid as sole carbon source. Twenty three genes of the Dit Island in *P. aeruginosa* are highly homologous to genes in *P. abietaniphilia* BKME-9 (62–91 % sequence identity of their protein product similarity); several orthologs are also found in *B. xenovorans* genome. The *P. aeruginosa* strain with acquired Dit Island could infect the CF patient and was able to establish a persistent infection lasting for years as well as grow in an environment rich in abietane diterpenoids. Thus the acquisition of new genetic elements, and consequently new traits, does not eliminate others, and the organism retains its ability to thrive in the widest possible range of environments by acquisition of novel metabolic capabilities through HGT and shaping the *P. aeruginosa* genome which is reflected in the genome plasticity of individual strains.

4.3 Comparative Functional Genomics

Comparative genomics provide a powerful tool to identify functionally important genomic elements. As more and more genomic information becomes available, further insight into the essential life processes of the organisms is obtained. Currently, database is available for about 70 genomes of *Pseudomonas* strains covering groups important in areas of human and animal health, agriculture as plant pathogens, plant growth promoters and biocontrol agents and biotechnologically important strains involved in degradation and bioremediation of pollutants in the environment and production of industrially useful products. Comparison of the genes involved in important subcellular features of different strains of *Pseudomonas* shows that only *P. aeruginosa* PAO1 produces outer membrane vesicles, and besides *P. aeruginosa* only two other strains, *P. syringae* pv. tomato DC3000 and *P. fluorescens* SBW25, have genes for synthesis of T3SS (Table 4.3).

As the functional differences exist both between the species of the genus *Pseudomonas* and strains within the species, it is important to examine if these functions are localized within particular region that are unique to the strain. Overall comparison shows that 4500 and 4400 genes accounting for more than 75 % genes in *P. aeruginosa*, the type species of the genus, are homologous to *P. putida* and *P. syringae*, respectively.

4.3.1 Metabolism, Transport and Regulation

Functional assignments of the products of predicted ORF were based on an identity or similarity of any ORF to products of characterized genes of *P. aeruginosa* or other bacteria and minimally the presence of conserved sequence motif defining a putative biological function. The ORF that were assigned different functions and their comparison in different strains are listed in Table 4.4. Transport of nutrients also involves major representation of 560 proteins (10 % of the genome). This

Table 4.3 Comparison of the genes involved in subcellular components (<http://www.pseudomonas.com>)

Feature	PAO1	PA14	PpKT2440	Pp W619	Pt PfO1	SBW25	Ppr Pf5	Pen L48	DC3000	PstA1501
Total number of genes	5688	5892	5516	5182	5722	5921	6108	5134	5481	4209
Outer membrane	189	176	144	133	131	165	163	140	121	78
Localization unknown	1318	1498	1397	1366	1484	1746	1581	1341	1679	998
Cytoplasmic membrane	1301	1328	1209	1201	1343	1491	1479	1171	1209	1014
Periplasm	209	179	153	153	184	211	210	155	172	114
Flagellar	18	17	16	16	17	17	16	16	17	18
Outer membrane vesicles	338	—	—	—	—	—	—	—	—	—
Cytoplasmic	2611	2642	2400	2292	2510	2712	2614	2278	2365	1893
Fimbrial	6	7	2	2	2	6	6	2	7	2
Host associated	6	—	—	—	—	—	—	—	—	—
Extracellular	90	69	47	37	70	72	61	49	73	30
T3SS	4	4	—	—	—	2	—	—	2	—

P. aeruginosa PAO1, PA14; *P. putida* KT2440, W619; *P. fluorescens* Pf01, SBW25; *P. protegens* Pf-5; *P. entomophila* L48; *P. syringae* pv. tomato DC3000; *P. stutzeri* A1501

Table 4.4 Distribution of genes for primary metabolism/functions of selected strains of *Pseudomonas* (<http://www.pseudomonas.com>)

Primary/metabolic function	PAO1	PA14	PpKT2400	PpW619	Pf- Pf01	PfSBW25	Ppr Pf5	Pen L48	DC3000	PstA1501
Intracellular trafficking, secretion, vesicular transport	175	200	123	128	134	145	149	142	140	94
Defence mechanism	78	78	63	54	65	61	80	65	57	58
Replication, recombination, repair	138	162	225	166	152	174	153	142	380	224
Energy production and conversion	329	333	296	286	294	284	308	260	237	262
Secondary metabolites, biosynthesis, transport	161	167	128	115	134	155	163	147	117	91
Chromatin structure and dynamics	3	3	2	2	3	4	5	2	1	1
RNA processing and modification	2	2	3	1	2	1	3	4	2	1
Cell motility	155	162	127	132	138	168	147	140	165	122
Cell wall/membrane/envelope biogenesis	267	272	267	257	308	277	300	275	277	185
Function unknown	519	204	445	423	486	427	523	443	421	333
Translation, ribosomal structure and biogenesis	205	546	188	189	199	195	216	196	200	185
Post translational modification, protein turnover	200	205	175	165	184	179	182	175	156	165
Transcription	492	510	441	421	465	522	561	401	379	239
Coenzyme transport and metabolism	215	215	186	183	199	196	221	187	178	148
Cell cycle control, cell division, chromosome partitioning	40	42	42	37	39	38	45	39	44	35
Amino acid transport and metabolism	498	500	186	445	498	595	599	480	460	298
Carbohydrate transport and metabolism	230	231	229	210	251	294	288	191	266	158
General function prediction only	633	640	565	524	620	613	682	547	553	417
Nucleotide transport and metabolism	108	111	92	93	97	234	104	175	83	80

(continued)

Table 4.4 (continued)

Primary/metabolic function	PAO1	PA14	PpKT2400	PpW619	Pf- Pf01	PfSBW25	Ppr Pf5	Pen L48	DC3000	PstA1501
Lipid transport and metabolism	235	238	181	177	202	100	248	97	180	146
Inorganic ion transport and metabolism	308	310	276	258	287	313	343	263	282	220
Signal transduction mechanism	344	350	332	328	363	397	404	324	366	282

P. aeruginosa PAO1, PA14; *P. putida* KT2440, W619; *P. fluorescens* Pf01, SBW25; *P. protegens* Pf-5; *P. entomophila* L48; *P. syringae* pv. tomato DC3000; *P. stutzeri* A1501

reflects the ability of *P. aeruginosa* to utilize variety of nutrients in diverse environment. Over 300 cytoplasmic membrane transport systems have been identified; about 2/3 are involved in transport of nutrients and other small molecules. *Pseudomonas putida* has a variety of transporters for mono-, di- and tricarboxylic acids but are relatively deficient in sugar transport. The lack of efficient sugar transport may be correlated with absence of an intact EMP pathway and its related reactions. Outer membrane proteins (OMP) in *Pseudomonas* are important as the cell surface serves to trigger response mechanism to the environment, transport of antibiotics, export of virulence factors and anchoring of the structures that mediate adhesion and motility. About 150 genes are predicted to encode OMP. These include the porins with specific function for transport of nutrients; the gated porins for transport of large molecules, e.g. siderophores-iron complex; and the porins involved in efflux pump or secretion systems.

Regulatory mechanism, the transcriptional regulators and two component regulatory systems are predicted to be encoded by 517 genes representing 9.3 % of the genome. Compared to other bacteria like *Helicobacter pylori*, *B. subtilis* and *E. coli*, larger component of regulator components corresponds to LysR, AraC, ECF- σ and two component regulator families. There exist a large number of proteins of the putative two component regulatory system with 55 sensors, 89 response regulators and 14 sensor response regulator hybrids. Such a system may be ideal for the organism to respond to changes in the surrounding environment.

Though *P. aeruginosa* have limited ability to grow on sugars, they can utilize a variety of other compounds as carbon and energy sources which provide for its versatility. A number of genes have been predicted that encode putative enzymes for β -oxidation. For example, 25 and 26 genes code for acetyl-CoA dehydrogenase and enoyl-CoA hydratase/isomerase, respectively. No other bacteria except *M. tuberculosis* contain such large number of these enzymes among organisms whose genome has been sequenced. β -oxidation genes are clustered with genes coding for other proteins which have related functions such as acyl-CoA thiolases, short-chain dehydrogenases, flavin containing monooxygenases or other oxidoreductases.

The whole genome comparison of *P. putida* W619 with other species of *Pseudomonas* shows that they share 3708 coding sequences (CD). In addition, 684 genes are shared among different strains of *P. putida*. Furthermore, 82, 47 and 108 CDS are uniquely shared between W619 and genomes of strain KT2440, GB1 and F1, respectively. Outside *P. putida* species *Pseudomonas entomophila* L48 shares maximum number of 110 genes with *P. putida* W619 and therefore considered closest to it. Furthermore, *P. putida* W619 has 170 CDS that have no hit (at E value of $<10^{-5}$) to any CDS from the sequenced species, indicating that these genes have originated probably from organisms outside *Pseudomonas*. Less number of CDS is shared with *P. syringae* group, *P. fluorescens* group, *P. stutzeri* and the least of two CDS with *P. aeruginosa* group.

Pseudomonas putida KT2440 shares a close evolutionary relationship with *P. aeruginosa* an opportunistic pathogen as well as with *P. syringae* that are plant

pathogens. The genome of *P. putida* KT2440 contains 1231 ORF, representing 22.7 % of total genome, that are absent in *P. aeruginosa*. This includes the novel operon for synthesis of cellulose as seen in *Agrobacterium tumefaciens* and *Rhizobium* species and is important for attachment of KT2440 to plant roots.

Among these, the other genes represent 226 conserved hypothetical proteins, 575 hypothetical proteins, 36 putative transposable elements, three phages, two toluene resistance proteins and 75 proteins involved in energy metabolism and type IV pilus biosynthesis. On the other hand, *P. aeruginosa* has 1281 ORF that are absent in *P. putida*. They encode 852 hypothetical proteins and 137 conserved hypothetical proteins, the key determinants of virulence and virulence-associated traits such as exotoxin A, elastase, exolipase, phospholipase C, alkaline protease, type III secretion pathway, two iron/manganese transporters and an operon for biosynthesis of rhamnolipids. All the virulence-associated genes are absent in *P. putida* KT2440 except for genes PP0168, PP1449 and PP0806 coding for adhesion protein which may be essential for seed colonization (Espinosa-Urgel et al. 2000). Homologs of these have also been identified in *P. fluorescens* that promotes plant-bacteria interactions (Dekkers et al. 1998).

Another virulence-associated gene that KT2440 shares with *P. aeruginosa* is quorum-sensing gene; however it does not produce detectable amount of homo-serine lactone, the signaling molecule. *Pseudomonas putida* KT2440 also possess 23 of the 24 genes involved in biosynthesis and regulation of alginate in *P. aeruginosa*. The only gene missing in *P. putida* KT2440 is all important *algM/mucC*, the transcriptional regulatory gene which renders the alginate gene nonfunctional. The loss of *algM* in *P. aeruginosa* results in a non-mucoid phenotype (Ohman et al. 1996). The 33.5 kb region spanning PP2815–PP2790 of *P. putida* KT2440 has 90 % similarity with genomic island PAGI-1 of *P. aeruginosa* isolated from sepsis and UTI infection (Liang et al. 2001).

Comparison with phytopathogens, *P. syringae*, *Ralstonia solanacearum*, *Xanthomonas campestris* and *Xylella fastidiosa* shows that *P. putida* KT2440 genome does not contain plant-related virulence traits such as type III secretion system as well as plant cell wall-degrading enzymes (Cao et al. 2001; Guttman et al. 2002). However, KT2440 does contain operons PP3790–PP3781 and PP2788–PP2777 related to biosynthesis of secondary metabolites (Bender et al. 1999). Comparative MLSA analysis of *P. fluorescens* of *gapA*, *gltA*, *gyrB* and *rpoD* indicated that this is a very diverse clade and many other species are included in this (Silby et al. 2009). Over all the number of orthologous genes identified as reciprocal best BLAST hits was low at 59.3 % of the CDS in strain Pf-5 and concentrates around the origin of replication. Unique genes comprised 22–27 % of CDSs in *P. fluorescens*. This is in sharp contrast to *P. aeruginosa* which shows only 1.4–8.2 % as unique genes (Mathee et al. 2008). Among the seven genomes of *P. fluorescens* strains having CDS in the range of 5333–6224 only 2789 CDS were found to be common in strains of *P. fluorescens* and the number of genes that were unique to each strain was high (Loper et al. 2012). Distribution of clusters of orthologous genes (COG) in different *Pseudomonas* spp is represented in

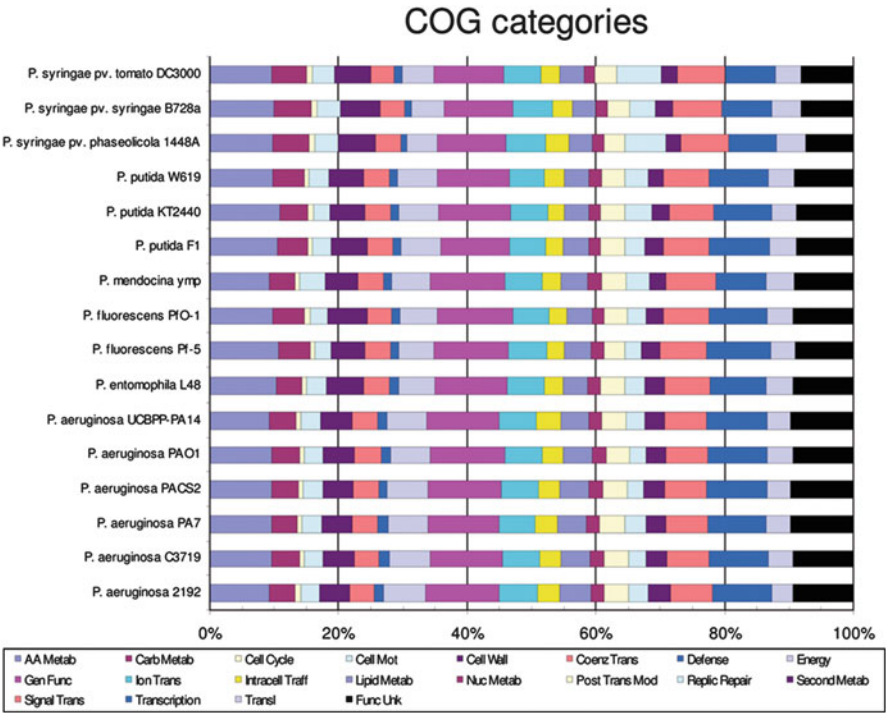


Fig. 4.5 Comparison of COG categories among different *Pseudomonas* spp. (Kiil et al. 2008)

Fig. 4.5. Nearly 90 % of gene functions have been assigned and for about 10 % function is unknown, i.e. the protein exists but its function is unknown.

4.3.2 Central Metabolic Pathway

Pseudomonads are well known for their metabolic versatility and share orthologs for transport and uptake of nutrients and their metabolism and regulation. *Pseudomonas* lack enzymes of EMP pathway and metabolize glucose and other hexoses via Entner-Doudoroff (ED) pathway through the formation of 6-phosphogluconate as the key intermediate giving rise to glyceraldehyde-3-phosphate and pyruvate. Thus only partial EMP is operative and *Pseudomonas* lack 6-phosphofructokinase, the key enzyme of the pathway. Genes encoding for fructose-1,6-biphosphatase (*fbp*; PP5040) and glucose-6-phosphate isomerase (*pgi*, two copies; PP1808, PP4701) which specify initial steps of gluconeogenesis and EPS biosynthesis have been identified in *P. putida* KT2440 but lack genes for aldolase-1-epimerase (*galM*) and glucose-1-phosphatase (*agp*). All the enzymes of the pentose phosphate pathway (PPP), TCA cycle, glycolate shunt and oxidative and electron transport chain are present for provision of energy as well as precursors and reduction of

power for biosynthetic reactions. Chemiosmotic gradient formed is the driving force for ATP synthesis by F-type ATP synthase. A variety of sugars, organic acids and amino acids are degraded by *Pseudomonas* and *Pseudomonas putida* has the broadest range of substrates utilized as sources of carbon and energy. *Pseudomonas putida*, *P. fluorescens* and *P. syringae* have genes encoding for extracellular enzymes, chitinases, proteases and lipases for degradation of polymers. They also share enzymes for utilization of plant-related products such as sucrose, maltose, trehalose and xylans. Two β -oxidation pathways are operative and genes for the synthesis of an extensive range of redox proteins, coenzymes and cofactors have been identified. *Pseudomonas aeruginosa* PA01 has as many as 25 genes for acyl-CoA dehydrogenase and 16 for enoyl-CoA hydratase/isomerase. Genes for β -oxidation are not clustered but may show clusters with various other proteins involved in the process. Though *P. putida* KT2440 does not undertake anaerobic metabolism/denitrification, it has two oxygen-independent coproporphyrinogen III oxidase genes (PP0141, PP5101), nitrite reductase complex (*nirB* and *nirD*; PP1705-1704) and several genes of fermentative metabolism such as lactate dehydrogenase (*ldhA*; PP1649) clustered with *gacS* (PP1650) coding for histidine kinase, phosphotransacetylase (*pta*; PP0774), formaldehyde dehydrogenase (*fdhA*; PP4960, PP0328, PP3970 and truncated PP1939) and an acetoin gene cluster (PP0550-0556).

4.3.3 Peripheral Metabolism and Bioremediation

Pseudomonas putida possess vast metabolic potential and are of interest as they can degrade variety of aliphatic long-chain alkanes, aromatic compounds and polycyclic compounds and their substituents as sources of carbon and energy. As such they are considered important in bioremediation of environment contaminated with hazardous chemical pollutants. Nutritional versatility is coupled with the production of biosurfactants that help in mobilizing hydrocarbons and nonaqueous phase liquids into an aqueous phase (Desai and Banat 1997). Besides this *P. aeruginosa*, *P. madocina* and *P. stutzeri* have been isolated from contaminated environment (Grimberg 1996; Loh and Cao 2008).

They have the capacity to adapt and survive in diverse niches by synthesizing unique membrane proteins, e.g. *P. syringae* has a large number of transporters for plant-derived sugars (Buell et al. 2003). *Pseudomonas putida* has more transporters than *P. aeruginosa* and uses these for transport of amino acids and aromatic compounds.

Though KT2440 are versatile in their metabolic pathways for degradation of aromatic compounds, it is unable to grow on aromatic hydrocarbons as sole source of carbon and energy. In contrast strain F1 is capable of growing on several hydrocarbons such as benzene, toluene, ethylbenzene and *p*-cymene and its acid *p*-cumate. Toluene degradation is featured by toluene dioxygenase operon *todABCDSE* and two gene regulatory component *todST* (Gibson et al. 1990; Lau et al. 1997). The F1 strain metabolizes *p*-cymene (*p*-isopropyl toluene) and *p*-

cumate using two pathways: in the first *p*-cymene is converted to *p*-cumate encoded by *cym* *AaAbBCDER* (Pput_2900-2905, 2908) and the second adjacently located is involved in the oxidation of *p*-cumate to isobutyrate, pyruvate and acetyl-CoA, *cmtAaAbAcAdBCDEFGHI* (Pput_2888-2899) (Eaton 1996, 1997). In addition, genomic sequence comparisons predicted universal existence of other aromatic catabolic pathways in all four *P. putida* strains including the protocatechuate (*pca*) and catechol (*cat*) and branches of β -ketoacidipate and phenylacetate (*pha*) pathways.

Genome of W619 contains two *mph* operons for degradation of 3-hydroxy-phenyl-propionate (HTT) (Ferrandez et al. 1997): one complete operon *mhpRABCDFET* for conversion of 3HTT to acetyl-CoA and the other incomplete operon comprising of three genes, the *mhpEFP*, which are conserved with *cym/cmt* pathway. This strain is unable to metabolize toluene or TCE. The operon *mhpRABCDFET* in *P. putida* W619 is flanked by truncated copy of IS1182 (Pput W619_1976-1977) and IS1382 (PputW619_1990) which are absent in KT2440, GB1 and F1. These mobile genetic elements are acquired by horizontal gene transfer. For the metabolism of long-chain and aromatic hydrocarbons, *Pseudomonas* synthesize a number of mono- and dioxygenases which incorporate one oxygen and two oxygen atoms into molecule and involve a number of coenzymes and cofactors. Besides *P. putida* KT2440 various oxygenases are also produced by strains of *P. aeruginosa* and plant growth-promoting rhizobacteria and other saprophytic strains for degradation of recalcitrant molecules. Homologs of the specific genes have been identified in different strains.

4.3.3.1 Chlorinated Compounds

Pseudomonas knackmussii B13 was first isolated in 1974 for degradation of chlorinated aromatic compounds (Dorn et al. 1974) and is important from point of view of bioremediation. Genomic annotation showed that B13 has a variety of pathways for degradation of monoaromatic hydrocarbons including chlorobenzoate, aminophenol, anthranilate and hydroquinol but not polyaromatic compounds (Reineke and Knackmuss 1979, 1988). Comparative genomic analysis revealed that B13 is closest to *P. denitrificans* and *P. aeruginosa*. B13 genome contains at least eight genomic islands (prophages and integrative conjugative elements, ICE) which are absent in closely related pseudomonads (Miyazaki et al. 2015). Being important in bioremediation of chloroaromatics, B13 was subjected to molecular breeding for transferring the capacity to degrade chloroaromatic compounds to other bacteria (Ravatt et al. 1998; Pieper and Reineke 2000). The mobile genetic element responsible has been identified as integrative conjugative element named ICE*clc* because it carries traits for degradation of chlorocatechol. It has a size of 103 kb and occurs as two copies in B13 genome. Sequence of ICE*clc* shows a composite nature of the region with *clc* genes, region for 2-aminophenol degradation and a ~50 kb region with gene synteny to other known ICE (Gaillard et al. 2006). The genes in this region are important in self-transfer.

Per the sequence analysis, the B13 genome measures 6,167,895 bp single circular chromosome containing 5753 predicted coding sequences (CDS). Strain B13 carries four copies of the 16S and 23S rRNA genes. Two identical copies of ICE clc lie at the 3' ends of two out of six genes for glycine-accepting tRNA (tRNA^{gly}) interspaced by 240 kb. The 16S rRNA gene sequence of B13 shows 99 % identity with that of *P. denitrificans* ATCC13867 and the two species are closely related. Phylogenetic analysis of concatenated set of amino acid sequences from 20 housekeeping proteins indicated that *P. knackmussii*, *P. denitrificans* and *P. aeruginosa* fall in a single clade.

Pseudomonas knackmussii B13 degrades 3- and 4-chlorobenzoate by multicomponent benzoate or toluate 1, 2-dioxygenase leading to formation of 3- or 4-chloro-3,5-cyclohexadiene 1,2 diol-1-carboxylic acid (CCCA). This enzyme has been characterized in *Acinetobacter* sp. ADP1 and *P. putida* WWO as BenABC and XylXYZ, respectively. CCCA is further metabolized to 3-,4-chlorocatechol by a dihydrodiol dehydrogenase (BenD/XylL) (Harayama et al. 1991). Single locus xylXYZL (PKB_2101-2104) on B13 chromosome shows 72–85 % identity to xylXYZ from pWWO (splP23099|XYLX_PSEPU) and 54–68 % to classical BenABCD system of *Acinetobacter* sp. ADP1 (splPO7769|BENA_AC1AD). The intermediates 3- and 4-chlorocatechol are degraded by ortho cleavage encoded by *clcABDE* loci (PKB_3275-3279 and PKB_3624-3628) (Schwien et al. 1988; Ravatn et al. 1998). Like other pseudomonads, the β -ketoadipate is converted into succinyl-CoA and acetyl-CoA, the intermediates of TCA pathway catalysed by *catFIJ* (PKB_2951-2953) (Harwood and Parales 1996).

The locus xylXYZL is preceded by a transporter (*pcaK*-like) and regulatory gene analogous to *xylS* (PKB_2099) to control the expression of xylXYZL in response to chlorobenzoate. Between *xylS* and xylXYZL lies a small CDS, PKB_2100 which encodes another transcriptional regulator. Next to xylXYZL loci lie genes for catechol to muconolactone pathway (i.e. *catR*, *catB*, *catC* and *catA*) (Miyazaki et al. 2015).

Genes for anthranilate dioxygenase (*antCBA*/Pkb_2111-2113) lie downstream of these but in opposite direction on ICE clc (Bundy et al. 1998). ICE clc also contains genes for meta cleavage of 2-aminophenol (Gaillard et al. 2006). Locus PKB_1742-1747 encoding phenol hydroxylase is homologous to *dmpKLMNOP* of *P. putida* CF600. This is preceded by another *catA* gene and *xylR/DmpR* homolog suggesting that B13 degrades phenol to *cis,cis*-muconate via catechol and proceeding further through ortho cleavage. B13 also codes for degradation of 4-hydroxybenzoic acid, hydroxyquinol and homogentisate. However, no specific homologous gene cluster homologous to polyaromatic hydrocarbon degradation such as biphenyl, naphthalene or phenanthrene has been found in B13 genomes (Miyazaki et al. 2015).

Comparison of the genome of *P. knackmussii* with that of *P. denitrificans* ATCC13867, *P. aeruginosa* PA01, *P. stutzeri* DSM4166 and *P. putida* KT2440 showed eight regions of genome plasticity referred as genomic islands, GI1–GI8 generally more than 20 kb in size, absent or different in other species. Their low GC ratio (61.5 %) compared to genome (66.0 %) suggested their horizontal acquisition.

GI4 and GI5 represent two copies of *ICEclc*, while GI1, GI3 and GI7 were predicted to be prophages 2, 3 and 5, respectively. The GI6 and GI8 don't contain phage-like genes but code for integrases of the tyrosine recombinase family (PKB_4426 and PKB_5453, respectively) located downstream of tRNA^{gly} gene (for GI6) and tRNA^{thr} gene (for GI8).

The GI2 is 22 kb and comprises of 21 CDS. Gene PKB_1177 located at one end of GI2 is homologous to IS Ppu10 transposase of *P. putida* (Ramos-Gonzalez et al. 2006). It also contains a large gene cluster (PKB_1181-1197) coding for lipopolysaccharide biosynthesis and export.

β-Proteobacteria, capable of degrading polychlorinated biphenyls, have been found to contain two ICE with nearly 100 % identity to *ICEclc*. One of these is 124 kb *ICEclc*^{LB400} found in *Burkholderia xenovorans* LB400. This has two insertions compared with *ICEclc*. The second *ICEclc*-like element was found in *Ralstonia* sp. strain JS705. The *ICEclc*^{JS705} has a 10 kb insertion of a gene cluster for a multicomponent dioxygenase and dihydrodiol dioxygenase to enable to metabolize monochlorobenzene in addition to 3-chlorobenzoic acid (Chain et al. 2006; Müller et al. 2003). A large number of genomes (more than 100) have been observed to carry sequences that bear homology *ICEclc* "core" region. In general, the integrase gene and core regions are separated by variable region of 20–60 kb with a highly variable gene content in a variety of organisms such as *Nitrosomonas europaea* C91, *Bordetella petrii*, *Achromobacter xylosoxidans* and *Acidovorax* sp. The traits that ICE carries include bleomycin resistance, mercury, polychlorinated biphenyls, 1-CBA, 2,5-di CBA degradation, streptomycin resistance, etc. (Gross et al. 2008). *Pseudomonas aeruginosa* CF18 isolated from CF patients in the USA and *A. xylosoxidans* isolated from CF patients in Denmark (Jakobsen et al. 2013) contain identical ICE, 91 kb in size having 99.9 % identity. This codes for bleomycin resistance and appears to be self-transmissible and plays role in chronic infection in CF patients.

4.3.3.2 Nutrient Uptake and Transport

Pseudomonads being very versatile in metabolism support a vast array of transporters to adapt to the variety of niches for their nutritional requirements and growth. *Pseudomonas putida* KT2440 codes for 370 membrane transport systems which accounts for about 12 % of the genome and is more than *P. aeruginosa*. ABC (ATP-binding cassette) transporters are the largest family with 94 paralogs and major part of it is devoted to amino acids as the plant root exudates are rich in amino acids. Besides they encode for transport of osmoprotectants and compatible solutes such as glycine betaine and organic acids including butyric acid which is the precursor for bioplastic synthesis. KT2440 also specifies for 23 member porin family of BenF/PhaK/OprD involved in uptake of aromatic compounds. Sugars like ribose use ABC system, while fructose is transported by one PTS system. KT2440 has two incomplete TRAP family dicarboxylic acid transporters (PP1167, PP1169) compared to four for *P. aeruginosa*. They also code for some specific proteins for ions like sulphate and metal ions like sodium, potassium, magnesium, etc. Iron binds the siderophore and pyoverdine and the genes are grouped in three

clusters (PP4243–PP4246, PP4319–PP4327 and PP4219–PP4223) as observed in both *P. putida* and *P. fluorescens*. Members of the genus *Pseudomonas* also encode an efflux system for removal of toxic molecules including heavy ions, secondary metabolites and antimicrobials. Efflux system has been well documented in *P. aeruginosa* particularly with respect to antibiotic resistance.

4.3.4 Regulation and Signal Transduction

Pseudomonas aeruginosa PA01 encodes a large number of regulatory proteins. Out of the total of 521, three hundred account for cytoplasmic membrane proteins and nearly 150 are outer membrane proteins. About 10 % of the genome of *P. putida* KT2440 is involved in regulation and signal transduction in response to the environment. This includes a number of sigma factors, viz. SigX, RpoD (σ^{70}), RpoN (σ^{54}), RpoS (σ^{38}), RpoH (σ^{32}), FliA (σ^{27}) and AlgT (σ^{22} , a homolog of RpoE in *E. coli*). *Pseudomonas aeruginosa* and *P. putida* encode for 22 sigma-54-dependent transcriptional regulators as compared to other related bacteria, which specify fewer such regulators (Martinez-Bueno et al. 2002). Overall KT2440 share 93 % homology with 450 regulators of *P. aeruginosa* PA01. They also code for LysR (PF00126) transcriptional regulator family, which is the largest paralog in KT2440 involved in diverse functions such as expression of proteins involved in aromatic metabolism, e.g. PcaQ(PP1713), CatR(PP3716), PobR(PP3538) and BenR(PP3159) are involved in regulation of metabolism of protocatechuate, catechol, polyhydroxybenzoate and benzoate, respectively (Cowles et al. 2000).

As soil bacterium, pseudomonads confront diverse environment and stresses such as osmotic shock, pH, desiccation, suboptimum growth temperature and toxic chemicals. To cope up with the situation, KT2440 has a number of genes coding for universal stress proteins (Dagley 1971), cold and heat shock proteins (Burger et al. 2000; Cornelis and Matthijs 2002) and starvation-related response (DiGiandomenico et al. 2002). Many of these are glutathione-S-transferases, often involved in detoxification of xenobiotics and heavy metal ions.

Plants act against intruders by producing oxygen radicals. *Pseudomonas putida* strains produce enzymes such as catalase, peroxidases and superoxide dismutase to counter against reactive oxygen species (ROS). *Pseudomonas putida* W619 encodes three superoxide dismutases: SodA, a Mn superoxide dismutase (Pput W619_4269); SodB, an Fe superoxide dismutase (Pput W619_0981); and SodC, a Cu/Zn superoxide dismutase (Pput W619_2485). The gene *sodC* is located on a genomic island (region 12) and is absent in strains of *P. putida*. Strain of *P. putida* W619 is also predicted to contain five catalases KatA (PputW619_472), KatB (PputW619_2032), KatE (PputW619_5113), KatG (PputW619_2235) and putative catalase (PputW619_2390) and two alkylhydroperoxidase reductases, AhpF and AhpC (PputW619_3186-3187); four additional putative alkylhydroperoxidase reductases (one putative AhpC, PputW619_1113, and three having an AhpD domain, PputW619_3104, PputW619_3108 and PputW619_3238); two thiol peroxidases PputW619_2803 and PputW619_3977); two glutathione

oxidoreductase (Gor, PputW619_3188) and glutaredoxin, PputW619_3239). The KatB and AhpD domain proteins are unique to *P. putida* W619 whose genes are located on three genomic islands (regions 9, 19 and 20). An hydroperoxide resistance protein (Ohr, PputW619_1469) is located adjacent to its organic hydroxide resistance transcriptional regulator (OhrR, PputW619_1470). A flavoprotein nitric oxide dioxygenase for detoxification of free radical nitric oxide has been identified and has an anaerobic nitric oxide reductase transcription activator Nor (PputW619_4379). Oxidative stress response system is controlled via complex regulatory network, the key regulator being inducible peroxide resistance protein, PerR. In *P. putida* W619, the PerR protein is encoded by Pput W619_2615, a LysR family transcriptional regulator that shares 61.5 % identity with PerR of *E. coli* K-12. Peroxide resistance protein, PerR, regulates the expression of *dps* (DNA-binding stress protein, Pput W619_4005), *fur* (the ferric uptake repressor, Pput W619_0702), *ahpCF* and *katA* (Catalase A) (Lan et al. 2010). Regulation of ROS-responsive gene might also be iron dependent and coupled to iron-binding proteins such as bacterioferritin (Bfr).The bacterioferritin α -subunit (bfr A; Pput W619_4721) is located next to catalase A (Kat A) in *P. putida* W619, and *P. putida* KT2440 (dos Santos et al. 2004) and the BfrB and Bfd-associated ferredoxin gene (Pput W619_1111-1112) are located adjacent to *ahpC* (Pput W619_1113).

4.3.5 Comparative Pathogenicity

4.3.5.1 *Pseudomonas aeruginosa*: An Opportunistic Pathogen

Pseudomonas aeruginosa, an opportunistic human pathogen, is capable of inducing disease in co-inhabitants such as nematodes, zebrafish, insects such as fruit fly, wax moth as well as other mammals (Rahme et al. 2000; Miyata et al. 2003; Gellatly and Hancock 2013). It is the major pathogen contributing to morbidity and mortality associated with cystic fibrosis (CF) of the lung and hospital-acquired infections. *Pseudomonas aeruginosa* is a difficult pathogen to combat with due to development of biofilms which enhance resistance to antimicrobials and involvement of three overlapping quorum-sensing (QS) systems, a T3SS, sigma factors and two component regulatory systems in controlling virulence and resistance. Transport system is important and about 150 outer membrane proteins and 300 cytoplasmic membrane proteins have been identified and a number of these are associated with antibiotic resistance and infection. Genes coding for T1SS, T2SS and T3SS and chemotaxis and motility have been identified. *Pseudomonas aeruginosa* PA14 shows greater virulence than PA01. PA14 has 322 more CDSs than PA01 genome and approximately 96.3 % of the DNA sequence found in PA01 is also found in PA14 and approximately 92.4 % of PA14 DNA sequence is found in PA01. There are 58 PA14 specific gene clusters (478 genes) and 54 PA01 clusters (234 genes) (Table 4.5).

The strain-specific gene cluster lies in regions of low GC content, i.e. probably the genomic islands (GI). The two strains differ with respect to the presence of an insertion of 107,911 bp in PA14 identified and characterized as two novel

Table 4.5 Characteristic features of core and accessory genome of *Pseudomonas aeruginosa*

Feature	Core		Accessory	
	Average	Range	Average	Range
Size (kbp)	5844		727	430–1192
% of total genome	89.7	86.4–93.3	11.1	6.9–18.0
Average G+C %	67.0		61.2	60.5–62.2
Total no. of genes	5316		608	348–1090
Gene length (bp)	990	72–13,029	939	51–17,019
Overlapping genes (%)	28.3		33.8	26.3–40.7
Transposases	7		6	3–14
Type I integron genes	0		2	0–10
ICE-associated genes	5		127	4–192
Predicted phage genes	18		124	19–271

P. aeruginosa pathogenicity islands (PAPI-1 and PAPI-2) in the genome of PA14, which are absent PAO1 (He et al. 2004). The 108 kb PAPI-1 and 11 kb PAPI-2 exhibit highly modular structures and contain many of the 313 PA14-specific ORFs. More than 80 % of the PAPI-1 DNA sequence is unique, and 75 out of the 115 predicted ORF products are unrelated to any known proteins or functional domains. PAPI-2 harbours 15 predicted ORFs and 8 of these are absent from strain PAO1. PAPI-2 carries genes encoding the protein ExoU and its chaperone, SpcU (Kulasekara et al. 2006). ExoU is a phospholipase effector protein secreted by the *P. aeruginosa* type III secretion system, a potent virulence factor in animal models. Other islands involved in ExoU functions are *exoU* islands A, B and C and acquisition of these by PAPI-2 enhances virulence while deletion of PAPI-2 results in reduced virulence. On the other hand, most of the genes within these islands are homologous to known genes present in other human and plant pathogenic bacteria. Importantly, many PAPI-1 ORFs are also found in several *P. aeruginosa* isolates from cystic fibrosis patients. The type III secretion system (T3SS) of *P. aeruginosa* secretes these toxins through a well-defined secretion system comprising the secretion apparatus (termed the injectisome), translocators, secreted toxins and regulatory components. Four type III secretory toxins or effector molecules, namely, ExoS, ExoT, ExoU and ExoY, have been identified in *P. aeruginosa* (Hauser 2009). ExoT and ExoY have minor role in pathogenesis and are expressed in both strains. While ExoS and ExoU are major exotoxins, generally only one ExoS or ExoU is expressed in a strain. ExoS, a 49 kDa exoenzyme, is a bifunctional toxin that exerts ADP-ribosyltransferase (ADPRT) at C terminal end and GTPase-activating protein (GAP) activity at the N terminal. Both activities have an effect on actin cytoskeletal organization, although ADPRT activity has greater role in pathogenesis. ExoT, a 53 kDa form of exoenzyme S with 75 % sequence homology to ExoS, also exerts GAP activity to interfere with cell morphology and motility. ExoY is a nucleotidylcyclase that increases the intracellular levels of cyclic adenosine and guanosine monophosphate, resulting in oedema formation. ExoU, which exhibits

phospholipase A2 activity and is 100 times more potent than ExoS, is the major pathogenic cytotoxin causing alveolar epithelial injury and macrophage necrosis. ExoU is encoded by *exoU* lying within an insertional pathogenic gene cluster named, *P. aeruginosa* pathogenicity marker PAPI-2. Adherence is the prerequisite for injection of type III toxins, and loss of pili and flagella required for adhesion and motility, respectively, affects pathogenicity of the organism. Other traits involved in pathogenicity are quorum sensing, biofilming and alginate production, phenazine biosynthesis and enzymes elastase, alkaline protease and phospholipase C. Genomic analysis of PA14 virulence has demonstrated that pathogenicity in this organism is both multifactorial and combinatorial process. Within a given isolate, virulence is multifactorial system in which several factors combine to result in an overall virulence phenotype. Additionally, when comparing different strains, virulence is combinatorial in that pathogenicity factors may behave differently and that distinct combinations or groupings of these determinants may result in comparable virulence phenotypes (Lee et al. 2006).

4.3.5.2 *Pseudomonas syringae*: A Plant Pathogen

Pseudomonas syringae is characterized by its ability to epiphytically colonize plant and cause disease. They are generally named as a pathovar of the plant species from which first isolated. They are grouped into more than 50 pathovars and considerable variations occur both between and within different pathovars of *P. syringae* (Sawada et al. 2002). Because of their pathogenicity towards crop plants, they have been subject of considerable interest. Three strains *P. syringae* pv. tomato DC3000, pv. phaseolicola 1448A and pv. *syringae* B728a whose complete sequences are available have been subject of discussion (Sarkar and Guttman 2004). Epiphytic fitness is important in the plant infection process and as such they should be able to withstand the stresses specific to epiphytic environment. Important fitness determinants are biofilms, osmoprotection, and iron uptake. Production of type IV pili and polysaccharide, alginate, has been associated with attachment and biofilm formation (Chang et al. 2007; Yu et al. 1999). However, a mannose-rich PsI (polysaccharide synthesis locus) expolysaccharide identified in *P. aeruginosa* may be involved in biofilm formation in *P. syringae* as all the orthologs are available in *P. syringae* pto genome (PsPTO_3529-3539). Another polysaccharide probably involved in *P. syringae* host interactions and biofilm formation is acetylated cellulose implicated in biofilm formation in *P. fluorescens* SBW25 (Spiers et al. 2003; Gal et al. 2003). Complete *wss* biosynthetic cluster is present in *P. syringae* pv. tomato DC3000 (PSPTO_1026-1034) and production of cellulose has been confirmed (Ude et al. 2006).

Oxidative stress due to reactive oxygen species (ROS) and ultraviolet radiation is overcome by quenching ROS as well as *ruAB*-encoded DNA polymerase for repair of UV damage (Sundin and Murillo 1999). *Pseudomonas syringae* pv. *syringae* B728 is less sensitive to UV irradiation as compared to Pst DC3000 although they share the same mechanism for detoxification of ROS and DNA repair. However the two genomes differ with respect to catalase isozymes; the

Pss B728a has five isozyme, while DC3000 has three. Also Pss B728a genome has two copies of *rulAB* operon (Feil et al. 2005).

Osmotic stress is mitigated by exopolysaccharide production and import of osmoprotective compounds like betaine. *P. syringae* pv. tomato DC3000 has two transport systems for uptake of osmoprotective compounds: the first *opuCABC* transporter (PSPTO_4575-4578) for uptake of betaine and choline (Chen and Beattie 2007) and the second BCCT family transporters, *BetT*, (PSPTO_5269) for choline transport only (Chen and Beattie 2008). Choline provides better osmoprotection and is connected to betaine by enzymes encoded by *betIBA* locus (PSPTO_0440-0441, 0443). Under iron-limiting conditions, siderophores help in uptake of iron. Expression of pyoverdine siderophore has been reported in *P. syringae* pv. tomato DC3000 under iron-limiting conditions. However, the siderophore yersiniabactin though expressed in B728a but does not contribute positively to fitness for disease (Jones et al. 2007).

The genome of *Psto* DC3000 has two plasmids of 73 and 67 kb (Buell et al. 2003). *Psto* DC3000 has a large number of genes with unknown functions and exhibits high level of duplication with 2735 genes (48 %) in 637 paralogous families. Genes for utilization of γ -aminobutyric acid (GABA) present in the plant cell apoplast have been characterized and include a permease gene, three GABA transaminase genes and a succinate dehydrogenase. Also the number of genes involved in sugar transport was higher than that of amino acid transporters as compared to *P. aeruginosa* PA01 and *P. putida* KT2440. These may be involved in transport of plant derived sugars, viz. xylose, arabinose and ribose. The genome of *Psto* DC3000 has 298 virulence-related genes encompassing type III secretion system, siderophores, phytotoxins, adhesins, extracellular polysaccharide and pectinolytic enzymes and detoxification of antimicrobials compared to 191 in *P. aeruginosa* PA01. Of these 65 genes were unique to *P. syringae* pv. tomato DC3000.

For the effective disease the bacteria must actively defeat the host defence response mechanism. *Hop* effector proteins are a critically important class of virulence factors (Nomura et al. 2005). Forty six families of *Hop* effectors and seven families of “helper” proteins facilitate transport of effector proteins through the T3SS that have been identified in *P. syringae* (Chang et al. 2005; Ferreira et al. 2006; Oh et al. 2007). The specific plant target sites for the effectors have been identified (Lin and Martin 2007; Fu et al. 2007). The T3SS helper proteins play a role in translocation process. *HopAK1* and *HopP1* share hair pin-like properties with *HrpZ* and *HrpW* and have a role in translocation of effector proteins into the plant cell (Kvitko et al. 2007). The helper protein *HrpH* has a lytic transglycosylase and facilitates the passage of type III pilus. They are also regulated by the *HrpL* regulon component, *ApbE* ortholog (PSPTO_2105) which is involved in pyridine biosynthesis in *S. typhimurium* and alcohol dehydrogenase (PSPTO_0834) which impacts the bacterial growth in *Arabidopsis* (Vencato et al. 2006). The nonpathogenic *P. syringae* Psy642 does not carry the T3SS or the conserved and exchangeable effector loci flanking *hrp/hrc* gene (Clarke et al. 2010). They are similar to other nonpathogenic strains but can colonize leaf surfaces and endophytic spaces of

the plants. Strain Psy642 encodes seven putative effectors including AvrE, ExoY and ExoU homologs and atypical T3SS in different genomic locations; genes *hrpK* and *hrpS* were absent from the cluster. Similarities between Psy642 and *P. fluorescens* SBW25 with respect to T3SS have been reported.

Phytotoxins

Pseudomonas syringae produce an array of phytotoxins which contribute to virulence. These are synthesized using non-ribosomal peptide synthases and polyketide synthases. The lipodepsipeptidic toxins, viz. syringomycin and syringopeptin, are involved in attachment and surface activity. The antimetabolite toxins, phaseolotoxin and tabtoxin, target specific metabolic pathways in the host. Coronatine produced by *Pseudomonas syringae* pv. tomato DC3000 acts by suppressing stomatal defence and salicylic acid-mediated defence mechanism and activates the jasmonic acid (JA)-signaling pathway (Zhao et al. 2003; Raaijmakers et al. 2006; Underwood et al. 2007).

Two additional loci encoding toxins, syringofactins and mangotoxin, have been identified. The locus *syfRABCD* (PSTO_2828-2832) encodes gene products for synthesis of six related lipodepsipeptidic compounds called “syringofactins,” SyrA–SyrF (Berti et al. 2007). Although linear in structure they exhibit properties similar to cyclic lipodepsipeptidic toxins and are required for surfactant activity and swarming movement.

The second loci identified in *P. syringae* pv. *syringae* UMAF-0158A encode synthesis of “mangotoxin” an inhibitor of ornithine N-acetyltransferase as a virulence factor and an enzyme of ornithine synthesis, which causes apical necrosis of mango. The gene orthologs have also been found in other *P. syringae* genomes, e.g. PSPTO_5452-5458 in DC3000 (Arrebola et al. 2007) and even *P. entomophila* L48, pathogenic to *Drosophila*. *Pseudomonas entomophila* encodes the non-ribosomal peptide synthase (PSEEN_0132), and the metalloprotease AprA and the flanking loci are highly orthologous to the biosynthetic cluster for mangotoxin production (Lindeberg et al. 2008). Orthologs of toxin complex (Tc) genes have also been identified in nematode symbionts of insects and indicate the diversity of toxins produced by *P. syringae*. Proteins with activities of chitinase, lipase and haemolysin implicated in bacterial pathogenesis of insects have also been predicted in *P. syringae* (Vodovar et al. 2006).

A total of 298 genes conferring different functions are involved in virulence and most of these (81 %) are shared by DC3000 and *Pph1448A*. Strain *Pph1448A* encodes 24 T3SS, 18 shared with DC3000 and 12 genes common to all *P. syringae* pathovars (Studholme et al. 2009). Two T3SS systems have been identified in 1448A, one of which has close ortholog in *P. aeruginosa* genome and has a role in interaction with eukaryotic cell. Strain PssB728a harbours unique genes such as cellulase family protein (Psr 4600) pectate lyase (Psr 0852), a xylanase (Psr 4508) and type I secretion system (Psr3075-77). A distinctive feature of PssB728a is the presence of ice nucleation gene (Psr1608). This serves as a template for formation of ice crystals on the cell surface. Another gene encoding antifreeze protein (Psr 0931) homologous to *AfpA* of *P. putida* GR12-

2 has been identified in PssB728a (Muryoi et al. 2004). Orthologs of ice nucleation and antifreeze proteins are absent from the genome of Pst DC3000.

All the three *P. syringae* genomes possess a well-characterized T3SS, annotated orthologs of the type II secretion components and a number of type I ABC transporters. Cryptic copies of type II and III pathways are found in *P. syringae* pv. phaseolicola 1448A. In *P. syringae* pv. tomato DC3000, a twin-arginine transport (Tat) pathway has been identified (PSPTO_5155-5157). This is involved in translocation of folded protein complexes across the outer membranes (Maillard et al. 2007). Disruption of Tat pathway in DC3000 results in reduction of virulence on *Arabidopsis* sp. Genes encoding components of type VI secretion system (T6SS) are present at two locations in *P. syringae* pv. tomato DC3000 and mutations in T6SS results in reduced virulence.

A coordinated network of regulatory control mechanism is necessary for transition from epiphytic to apoplastic stage. This involves an array of alternate sigma factors and quorum-sensing signals (Quinones et al. 2005). Factors controlling expression of acyl homo-serine lactone signal (AHL) are integral to quorum sensing and have two regulators, AefR and PsrA, for upregulation and downregulation of AHL biosynthesis, respectively, in both DC3000 and PssB728a (Chatterjee et al. 2007). Genes regulated by HrpL have been comprehensively characterized in all the three strains (Ferreira et al. 2006; Chang et al. 2005; Vencato et al. 2006). Expression of HrpL is regulated by NtrC-like activators HrpR and HrpS (Lan et al. 2006). HrpL-binding sites have since been incorporated into *P. syringae* genome annotations and represent a valuable information.

Two strains T₁ and DC3000 of *Pseudomonas syringae* pv. tomato show high degree of similarity but significant differences have been noted in type III effector repertoire. The two strains also differ with respect to host specificity. *Pseudomonas syringae* pv. tomato T₁ causes disease only on tomato, while the DC3000 cause disease on *Arabidopsis thaliana*, the model organism besides cauliflower and tomato. Comparison of four genomes of *P. syringae* pv. tomato T₁ and DC3000, *P. syringae* pv. syringae B728a and *P. syringae* pv. phaseolicola 1448A shows that 4271 proteins are shared between all the four genomes and thus represent the conserved housekeeping gene products and virulence gene products underlying adaptation to the plant host. A total of 471 proteins are shared between strains T₁ and DC3000 and are absent from Pss B728a and Psp 1448A. A total of 757 proteins are unique to T₁. Out of these 511 are hypothetical proteins, 79 proteins can be classified as related to mobile genetic elements and 167 are proteins with other predicted functions that may have potential involvement in host specificity. DC3000 genome also contains 196 proteins with similar functions among the 740 unique genes for this strain. The repertoires of T3SS effectors of strains T₁ and DC3000 are diverse particularly with respect to *A. thaliana* infection. The effectors present in strain T₁ and absent in DC3000 could lead to gene for gene resistance of *A. thaliana* to T₁, while effectors present in DC3000 but absent in T₁ may account for the inability of T₁ to suppress ETI (effector-triggered immunity) or PTI (pathogen- or microbe-associated molecular pattern (PAMP)-triggered immunity). *Pseudomonas syringae* pv. tomato T₁ also lack genes for synthesis of

coronatine and a subset of T3E. Despite the high degree of similarity, the two strains differ with respect to effector repertoire. They share only 14 effectors while 15 are present in only in DC3000 and 11 only in T1 genomes.

4.3.5.3 *Pseudomonas entomophila*: An Insect Pathogen

Pseudomonas entomophila is an entomopathogenic species first isolated from fruit flies and is closely related to the saprophytic soil bacterium *Pseudomonas putida*. It was later characterized as a natural pathogen of *Drosophila* larvae and adults (Vodovar et al. 2005). *Pseudomonas entomophila* can also effectively infect and kill *Bombyx mori* and *Anopheles gambiae*, which makes it a potential biocontrol agent. Results of genomic sequence of *P. entomophila* suggest that this strain is a ubiquitous, metabolically versatile bacterium that may colonize diverse habitats, including soil, rhizosphere and aquatic systems, and closely related to *P. putida* KT2440 and finally included in the genus *Pseudomonas* (Mulet et al. 2012; Vodovar et al. 2006). Phenotypically *P. entomophila* cells are Gram-negative rods, motile by one polar flagellum, strict aerobe and catalase and oxidase positive and produce a fluorescent pigment but no pyocyanin. Interestingly, the colonies exhibit a strong haemolytic activity on blood agar plates and a significant protease activity on milk casein. It is also positive in the gelatin hydrolysis.

However, in contrast to the *P. putida* genome, the *P. entomophila* genome contains many genes that are predicted to be important for virulence towards insects. Notably, *P. entomophila* could secrete many degradative enzymes (proteases and lipases), putative toxins and secondary metabolites (Vodovar et al. 2006). Similar factors have been shown to play a key role in the virulence of other entomopathogenic bacteria like *Photorhabdus* and *Xenorhabdus* sp. The *P. entomophila* chromosome (Vodovar et al. 2006) contains 5,888,780 nucleotides with a GC content of 64.2 %. It is of an intermediate size compared to the other sequenced *Pseudomonas* genomes and 1002 genes are unique to *P. entomophila*. Close relationship between *P. entomophila* and *P. putida* is also indicated by the presence 70.2 % of *P. entomophila* gene orthologs in *P. putida* and 96 % are in synteny. Complete genome sequence of *P. entomophila* bears similarity to other pseudomonads. Genome of *P. entomophila* contains a large set of genes involved in the adaptation to multiple carbon sources as *P. entomophila* is a metabolically versatile bacterium capable to survive in the soil, rhizosphere and water (Almeida et al. 2009). Notably, the *P. entomophila* genome contains several genes that encode proteins with hydrolytic activities such as chitinases, lipases, proteases and some hydrolases with undefined activities. In addition, the *P. entomophila* genome harbours determinants for the catabolism of various aromatic compounds and long-chain carbohydrates making it potentially useful for bioremediation. *Pseudomonas entomophila* has an elaborate transport system involving more than 535 transporters and a high number for its wide adaptability in natural environment. However, in contrast to phytopathogen, *P. syringae*, the genome of *P. entomophila* lacks genes encoding enzymes for degrading plant cell walls. This is consistent with the fact that this is not pathogenic for plants (Vodovar et al. 2006) but may protect plants against insects and fungal diseases (Vallet-Gely et al. 2010).

Five gene clusters are associated with synthesis of secondary metabolites. Four of these are involved in non-ribosomal peptide synthase (NRPS) for synthesis of three different lipopeptides and a polyketide. One gene cluster is involved in NRPS synthesis of HCN which is predicted to have no significant role in insect pathogenicity (Vallet-Gely et al. 2010). One of the four gene clusters is involved in *Pseudomonas* virulence factor (PVF) synthesis by NRPS (*pseen0131*, *pseen0132*, *pseen0133*). A new gene product, named entolysin having haemolytic and surfactant activity, is synthesised by another NRPS encoded by *etlA* (*pseen3332*), *etlB* (*pseen3044*) and *etlC* (*pseen3045*) and has also been identified to play a crucial role. Entolysin is a cyclic lipopeptide containing 14 amino acids and 3-C₁₀OH fatty acids (Vallet-Gely et al. 2010). The loci *etlA*, *etlB* and *etlC* in *P. entomophila* are analogous to loci *psaA*, *psaB* and *psaC*, respectively, encoding putisolysin in *P. putida*.

Although HCN and most of the secondary metabolites encoded by *P. entomophila* are not essential for its virulence, these molecules may be involved in biocontrol (Haas and Defago 2005) such as killing of nematodes and suppressing microbial competitors in the soil (de Bruijn et al. 2007; Neidig et al. 2011; Li et al. 2013). The overall pathogenesis of *P. entomophila* depends upon (1) the ability to enter and persist in the gut and (2) the excretion of toxic substances that disrupt the host physiology. *Pseudomonas entomophila* has so far been found to be pathogenic for three orders of insects from Diptera (*Anopheles gambiae*, *D. melanogaster*), Lepidoptera (e.g. *Bombyx mori*, *Galleria mellonella*) and Coleoptera (e.g. *Sitophilus oryzae*). Most of the studies on pathogenesis of *P. entomophila* have been on adults and larvae of *Drosophila*. Genes encoding for TccC-type insecticidal toxin are particularly striking as they are found in entomopathogenic bacteria such as *Photorhabdus luminescens* and *Xenorhabdus nematophila* (Hinchliffe et al. 2010) and are absent in the genomes of other *Pseudomonas* (Vodovar et al. 2006). The *P. entomophila* genome encodes other proteins more distantly related to TccC- and TcdB-type insecticidal proteins. Proteases also contribute to the virulence and *P. entomophila* encodes three serine proteases (*pseen3027*, *pseen3028*, *pseen4433*) and one alkaline protease (*pseen1550*) absent from *P. putida*. The latter is a homolog of AprA which has been shown to be involved in virulence in other bacteria by protecting against the immune response and degrading of host tissues (Miyoshi and Shinoda 2000). AprA has been shown to be the most abundant protein in *P. entomophila* supernatant (Liehl et al. 2006).

A number of genes coding for cell surface associated factors allowing adhesion and colonization also contribute to pathogenesis and important among these are genes coding for filamentous hemagglutinin, a surface adhesion protein and the amyloid curli fiber.

The T3SS secretion system which is required for pathogenicity of higher organisms is absent in *P. entomophila* and, however, possesses a single locus containing the conserved core genes of T6SS proteins as well as several T6SS homolog proteins (VgrG and Hcp) dispersed in the genome. Notably, the components of the T6SS (Vgr, Rhs and Hcp proteins) are the most abundant

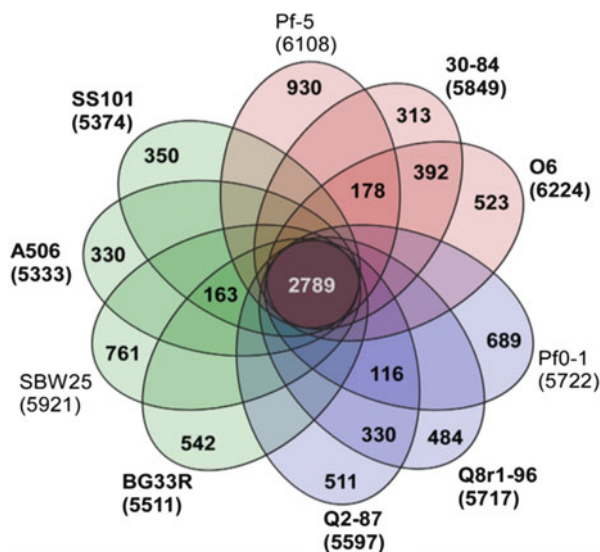
proteins excreted in the supernatant of this bacterium. In the absence of T3SS and T4SS, the T6SS system actively participates in *P. entomophila* virulence and insect-bacterium interaction (Opota et al. 2011; Sarris and Scoutica 2011). Two-component system GacS/GacA plays a key role in pathogenicity by controlling putative virulence factors and AprA, a secreted protease to escape the immune system of the fly. The GacS/GacA regulates its production and requires two small RNAs and two RmsA-like proteins. GacA is the response regulator of the GacS/GacA system and *gacA* mutants are defective in the secretion of protease and haemolysin (Vodovar et al. 2006). Mutations in the gene *pseen3045* and *pseen3042* affected an NRPS and an ABC transporter component, respectively.

4.3.6 *Pseudomonas fluorescens*: Commensal and Plant Growth Promoter

Pseudomonas fluorescens is group of plant commensals occupying rhizosphere and phyllosphere and is an effective antagonist. They are important biocontrol agents and promoters of plant growth by suppression of diseases by synthesis of toxic metabolites against phytopathogenic fungi and bacteria (Mavrodi et al. 2006; Pierson and Pierson 2010), enhancing nutrient availability for plant growth and production of phytohormones. They are known to produce polyketides 2,4 diacetylphloroglucinol, pyoluteorin and rhizoxin; the chlorinated tryptophan derivative, pyrrolnitrin; and hydrogen cyanide formed by oxidation of glycine (Howell and Stipanovic 1980; Nowak-Thompson et al. 1994; Weller 2007). Gene clusters for each of these metabolites have been identified in *Pseudomonas fluorescens* strains (Gross and Loper 2009). The phylogenetic tree of completely sequenced pseudomonads showed that *P. chlororaphis* GP72, an effective biocontrol agent, was most closely related *P. fluorescens* and closely related to *P. aeruginosa* M18 than to other strains of *P. aeruginosa*. This may be attributed to the fact that GP72 and M-18 have rhizospheric origin. As for typical *Pseudomonas*, all these strains lack 6-phosphofructokinase the key enzyme of EMP pathway, thus rendering it nonfunctional for sugar metabolism. In addition to these *Pseudomonas chlororaphis* subsp. *aurantiaca* BL915 has a locus for biosynthesis for 2-hexyl-5 propyl-alkylresorcinol (Nowak-Thompson et al. 2003). Similar loci have been identified in the genomes of *P. chlororaphis* strains 06 and 30.84 and can contribute to suppression of fungal and bacterial pathogens. A view of the spectrum of ecological, metabolic and biochemical characteristics shows that their diversity also extends to genomic variability and only 25–35 % of the genome of each strain is composed of the core genome shared by all members of the genus. Comparative genomic study of four strains of the *Pseudomonas fluorescens* group, viz. *P. protegens* Pf5 (*P. fluorescens* Pf5) and *Pseudomonas fluorescens* strains SBW25, Pfo-1 and WH6, shows tremendous diversity of these bacteria. Members of *P. fluorescens* group, representing plant growth-promoting rhizobacteria, show a number of strong features for plant growth promotion such as adaptability to environmental stresses, rhizosphere fitness and diverse metabolic potential (Loper

et al. 2012). Among the four strains known for plant growth promotion, *Pseudomonas chlororaphis* GP72 has strong antifungal activity towards phytopathogens, e.g. *Pythium ultimum*, *Colletotrichum lagenarium*, etc. (Liu et al. 2006; Huang et al. 2010; Shen et al. 2013); *Pseudomonas protegens* Pf-5, isolated from cotton rhizospheres, that suppresses damping-off caused by *P. ultimum* (Howell and Stipanovic 1980); *Pseudomonas aeruginosa* M-18, a rhizospheric isolate of sweet melon showing antifungal activities; and a nonfluorescent strain, *Pseudomonas stutzeri* A1501, isolated from rhizosphere of rice for its nitrogen-fixing capacity (Qiu et al. 1981; Vermeiren et al. 1999), have been characterized. The PGPR colonize the rhizosphere and have certain biocontrol activities through production of antimicrobials such as phenazine derivatives, pyoluteorin, pyrrolnitrin, 2,4-diacetylphloroglucinol (DAPG), cyclic lipopeptide biosurfactants and HCN. The genome sizes of the four species vary from 4.6 to 7.1 Mb with *P. stutzeri* A1501 having the smallest size as compared to others. Genomes of all the four strains contained type I, type II, type IV, type V and type VI secretion systems as well as chaperone-usher secretion system and twin-arginine translocation system. Genome of PAM18 also contained the type III secretion system, the key virulence factor in pathogenic *P. aeruginosa* strains. Genomic comparison of the four species showed that there were only 602 genes conserved among the four species with number of unknown common traits related to plant growth promotion; several features of genomes are presented in Table 4.1. On the basis of their relatedness, they could be grouped into three subclades. Of the 2789 core genes, only 20 are specific for *P. fluorescens* group, while the rest 2769 have orthologs in at least one of other sequenced genome of *Pseudomonas* spp. The 20 core genes include biofilm formation, hypothetical or conserved hypothetical proteins and regulation. The small number of core genes is attributed to diversity of strains within the *P. fluorescens* group and highly plastic nature of their genomes. Besides they also have a large pan-genome comprising of 13,872 CDS, which is larger than the pan-genomes of *P. syringae* and *P. aeruginosa*. Of the 13,872 pan-genome CDSs of *P. fluorescens* group, 5798 have no orthologs in other genomes of *Pseudomonas* spp. This indicates their horizontal acquisition from other taxa. Within the subclade strains share 69–90 % of their predicted proteomes, while strains in different subclade share only 64–73 % of their proteomes. Thus the core genomes for each subclade range between 3729 and 4188 CDSs which are much larger than the core genome for the whole group (Loper et al. 2012). They show high level of synteny around the origin of replication for the strains within the subclades but very little synteny between genomes of strains of different subclades. Three genomes of strains *P. protegens* Pf5, *P. chlororaphis* 30-84 and *P. chlororaphis* 06 of subclade 1 share 73 genes that are not present in any other sequenced *Pseudomonas* genome. These genes encode biosynthesis of pyrrolnitrin and insect toxin fit D. Three genomes of subclade 2 share 38 genes that are not present in any other genome of sequenced *Pseudomonas* and genomes of subclade 3 have 87 such genes that are shared among them and are not present in any other genome. These include genes for pili biosynthesis, type III secretion system and ribose metabolism. The comparison of the 10 genomes of *P. fluorescens* group shows about 300–900 (6–15 %)

Fig. 4.6 Genomic diversity among 10 strains of *P. fluorescens* falling in three subclades I, II, III represented by red, blue and green coloured ovals, respectively (Loper et al. 2012)



genes are unique to strains (Silby et al. 2009). A large number of strain-specific genes and large-sized pan-genomes indicate high level of genomic and biological diversity in *P. fluorescens* group (Fig. 4.6).

Novel natural products contributing to biocontrol of phytopathogenic fungi are phenazines, HCN, chlorinated tryptophan derivative pyrrolnitrin and the polyketides 2,4-diacetylphloroglucinol, rhizoxin and pyoluteorin (Mavrodi et al. 2006; Gross and Loper 2009; Chen et al. 2015). Gene clusters for these have been identified. In addition to these *Pseudomonas chlororaphis* subsp. *aurantiaca* BL915 genome has locus for synthesis of 2-hexyl-5-propyl-alkylresorcinol which exhibits mild antifungal and antibacterial activity; similar loci have been identified in the genomes of *P. chlororaphis* O6 and 30-84. The cyclic lipopeptides (CLP) composed of a cyclic oligopeptide with a lipid tail are produced by *Pseudomonas* sp. and show surfactant, antimicrobial, antipredation and cytotoxic properties. Genes coding for production of CLP orfamide A and derivatives of rhizoxin and traits such as LlpA bacteriocin and FitD insect toxin have identified on the specific region of the genome of *P. protegens* (Raaijmakers et al. 2010; Gross et al. 2007). The chain length and composition of lipid and number and configuration of amino acids in the peptide account for diversity of CLP. These compounds are synthesized by non-ribosomal peptide synthases (NRPS). Orthologs of genes coding for CLP massetolide A and viscosin lie at two different locations in genomes of *P. fluorescens* SS101 and SBW25, respectively, while for production of CLP orfamide A, there is a single gene present in strain Pf 5 genome. Gene clusters for CLP biosynthesis have been identified in the genomes of BG33R and PfO-1. The phenotypes of strain BG33R exhibit swarming mobility, haemolytic activity and surfactant activity. The predicted structure of CLP produced by BG33R is a 9 amino acid peptide similar to massetolide or

pseudophomins A and B, while the CLP produced by PfO-1 is distinct from all others and is an 11-amino acid peptide (Pedras et al. 2003).

The fluorescent pigments produced by *Pseudomonas* are a diverse class of siderophores, pyoverdine and pyochelin which help in acquisition of iron and rhizosphere adaptability. These have full complement of genome involved in biosynthesis, utilization and regulation of pyoverdine iron acquisition system and are distributed in three to seven clusters in the genomes of these organisms (Visca et al. 2007). The genomes of GP72, Pf-5 and M-18 also contained the complete Pvd biosynthetic gene cluster. In addition, Pf 5 and M-18 contained genes encoding another siderophore pyochelin (Pch) with antifungal activity (Kloepper et al. 1980; Meyer 2000). Many pseudomonads also produce secondary siderophore pseudomonine synthesized by NRPS pathway used for the synthesis of two other siderophores, acinetobactin and anguibactin (Wuest et al. 2009). Loper et al. (2012) have identified many orphan gene clusters, i.e. the loci with sequences of secondary metabolism genes but without any known biosynthetic product. Eight orphan clusters have genes for NRPS, two genes for polyketide synthases (PKS) and one hybrid NRPS-PKS. All strains except Pf-5 have clusters homologous to *pvfABCD* which contains an NRPS-encoding gene and is required for biosynthesis of putative signaling molecule in *P. entomophila* (Vallet-Gely et al. 2010). Three of the NRPS-containing orphan gene clusters in newly sequenced genomes are likely to encode the biosynthesis of secondary siderophores. Many other gene clusters for secondary metabolites, e.g. phenazines, 2,4-diacetylphloroglucinol, 2-hexyl-5-propyl-alkylresorcinol and achromobactin, are present in conserved locations within the genomes of closely related strains of the subclade and may have been acquired recently in the evolutionary history. Although *Pseudomonas* spp. produce a variety of secondary metabolites, only a few are synthesized via NRPS and PKS pathways. The secondary metabolites serve important function and are expected to role in ecology of these bacteria and interaction with soil- or plant-associated microorganisms.

Each of the 10 genomes of *P. fluorescens* strains sequenced has 2–7 bacteriocins; these include genes for many diverse bacteriocins including pyocins S1/2/3/AP41, S5 and colicin M-like bacteriocins and lectin-like Llp bacteriocins (Parret and De Mot 2002; Barreteau et al. 2009). Strain A506 has a region related to coding for microcin B17 production in Enterobacteria. The bacteriocins have narrow spectrum of activity and may contribute for control of *Pseudomonas syringae*. Genes coding for bacteriocin are clustered with genes including immunity, forming prototypic toxin-antitoxin gene pairs. Strains differ widely with respect to number and type of bacteriocin produced.

4.3.6.1 Phytohormones

Pseudomonads associated with plants influence plant growth by modulating plant regulatory mechanisms. Indole-3-acetic acid (IAA) produced by plant-associated bacteria has profound effect on growth and development of plants by controlling physiological processes (Lugtenberg and Kamilova 2009; Spaepen et al. 2007). Genes encoding tryptophan-2-monooxygenase (IaaM) and indole-3-acetamide

hydrolase (IaaH) which convert tryptophan into IAA have been detected in *P. chlororaphis* strains 30-84 and 06. Strains 30-84, 06 and Pf-5 also carry genes for degradation of plant hormone and antimicrobial metabolite phenyl acetic acid (PAA) and utilize it as sole source of carbon in a manner similar to *P. putida* U (Olivera et al. 1998).

Pseudomonas putida also produces IAA for plant growth promotion and strain W619 is most efficient producer of IAA in comparison to other endophytic bacteria (Taghavi et al. 2009). Consistent with this, *P. putida* W619 encodes two tryptophan-dependent pathways, one via tryptamine and indole-3-acetaldehyde that require tryptophan decarboxylase (PputW619_2223), an amine oxidase (PputW619_0482) and an indole-3-acetaldehyde dehydrogenase encoded by many putative genes, PputW619_0192/ 0213/0597/2257/2639/2872/2926/2546/3767. This pathway is also present in *P. putida* KT2440, F1 and GB1. Alternative pathway of conversion tryptophan to IAA via tryptophan 2-monooxygenase (PputW619_1175/4820) is analogous with *P. fluorescens*.

Aminocyclopropane-1-carboxylic acid (ACC) is the precursor of ethylene. Stressed plants accumulate ethylene, which inhibit root elongation and accelerate abscission, aging and senescence. Rhizobacteria producing enzyme ACC deaminase (AcdS) for conversion of ACC into ammonia and α -ketobutyrate, produce lower the levels of ethylene thus stimulating growth of roots. Only strain Q8r1-96 carries *acdS* gene. This gene is absent in *P. putida* W619, KT2440, GB1 and F1 (Glick et al. 2007).

Rhizobacteria also produce volatile compounds acetoin (3-OH-2-butanone) and 2, 3-butanediol to enhance plant growth (Ryu et al. 2003). *Pseudomonas fluorescens* and *P. putida* don't contain genes *budABC* identified in endophytic *Enterobacter* sp 638 (Taghavi et al. 2010). Genome of *P. chlororaphis* 06 contains a gene for acetoin reductase and produces 2,3 butanediol normally produced during mixed acid fermentation by Enterobacteriaceae. Genomes of other strains in subclade 1 also contain gene for acetoin reductase. Orthologs of gene *ilvBN* encoding α -acetohydroxyacid synthase were detected in all the strains of *P. fluorescens* (Loper et al. 2012). Six strains of *P. fluorescens* also carry *aco* gene for acetoin dehydrogenase (AoDH) enzyme complex that converts acetoin to acetaldehyde and acetyl-CoA. A cluster of four genes coding for regulator, protein AcoR and AcoABC representing proteins E1 α , E1 β and E2 subunits of AoDH is present in these genomes. Four strains (Pf5, 30-84, Q8r1-96 and Q2-87) also have gene *acoX* and 2,3-butanediol dehydrogenase gene and *bdh* for catabolism of 2,3-butanediol and acetoin. Genes *acoXABC adh* involved in the catabolism of acetoin and 2,3-butanediol to central metabolites have been identified in *P. putida* F1 (Pput_0595-0591), GBI (P put GBI_0601-0593) and KT2440 (PP_0556-0552). However these genes were absent in *P. putida* W619 (Wu et al. 2011).

Each of 10 strains of *P. fluorescens* studied by Loper et al. (2012) has a conserved cluster for the exoprotease, AprA. AprA has a role in biological control of fire blight disease of pear and apple. The cell wall of fungi is primarily composed of chitin and *Pseudomonas fluorescens* strains producing chitinase that hydrolyses cell wall of fungi, thus contributing to the biological control of fungal diseases. Two

chitinase genes in the genome have been identified. Gene pectate lyase for hydrolysis of pectin is present in *P. fluorescens* strain SBW25- and absent in other genomes (Nikaidou et al. 1993). Novel gene clusters were identified in each strain, and the mechanisms with which they interact with their cohabitants, host plants and other organisms need to be further explored (Loper et al. 2012).

4.3.6.2 Commensal Lifestyle

Pseudomonas fluorescens are commensal and colonize plant surfaces and thus possess characteristics such as iron acquisition and stress tolerance. Pseudomonads are known to use siderophores produced by other microorganisms as sources of iron and are fit to survive in environment with limited iron. Ton B-dependent outer membrane receptor proteins are responsible for uptake of each ferric-siderophore complex and play an important signaling role in the bacterial cell. Genome of *P. protegens* Pf-5 contains 45 genes for putative Ton B-dependent receptors, and sequences of 28 genes are similar to known receptors of ferric-siderophore complexes in other organisms including six putative receptors for pyoverdines, one in pyoverdine biosynthetic gene cluster and four distributed widely over the genome. *Pseudomonas protegens* has multiple receptors for hydroxamate siderophores and catechol siderophores. Multiplicity of pyoverdine receptors may confer to the ability to utilize a broad spectrum of these compounds. Like Pf-5 other *Pseudomonas* also have genes coding for putative Ton B-dependent proteins in their genome. *Pseudomonas protegens* Pf-5 neutralize superoxide (O_2^-) and H_2O_2 in the rhizosphere, two superoxide dismutase (SoD) and six catalases. Besides there are 10 peroxidases (three cytochrome C peroxidases, three glutathione peroxidases, two DyP-type peroxidases, a thiol peroxidase and an alkyl peroxidase). All these peroxidases are not unique to Pf-5 and are shared by other pseudomonads (Storz and Imlay 1999).

Pseudomonas protegens Pf-5 lacks the virulence factors found in plant pathogen; *P. syringae* and does not have genes for virulence factors such as phytotoxins, cellulases, pectinases or pectin lyases involved in cell degradation and type III secretion system. However, *P. fluorescens* SBW25 has a 20 kb gene cluster resembling type III secretion system (Preston et al. 2001) which are probably involved in colonization of sugar beet rhizosphere and its inactivation affects the efficiency to colonize rhizosphere. Besides T3SS in *P. fluorescens* may function in defence of bacteria against predation and competition in their natural habitat.

4.3.6.3 Biocontrol Activities

Biocontrol of phytopathogens is an important activity of PGPR. Biocontrol activity is mediated through the production of variety of antibiotics and secondary metabolites. Phenazines produced by *P. fluorescens*, *P. chororaphis* and *P. aureofaciens* play a key role in biocontrol (Mavrodi et al. 2010; Haas and Defago 2005). Clusters of phenazine compound biosynthetic genes were present in genomes of GP72 and M18, but the genes differed between the two species. The GP72 genome contained *phzO*, encoding an aromatic monooxygenase that converts phenazine-1-carboxylic acid (PCA) to 2-hydroxy phenazine (2OH-PHZ), whereas

the M18 contained two *phz* gene clusters and one set of modified *phzMS* genes; *phzM* codes for putative S-adenosylmethionine-dependent N-methyl transferase and *phzS* codes for putative flavin-dependent hydroxylase. These two enzymes participate in the conversion of PCA to PYO (pyocyanin), the virulence factor for *P. aeruginosa* infection of CF patients. However, M18 does not produce detectable level of pyocyanin (PYO) at 28 °C as the regulation of gene *phzM* and its regulatory genes *lasI* and *ptsP* are temperature dependent. It is assumed that the biocontrol activity of M18 is due to PCA (Huang et al. 2009). Another important antibiotic produced by Pf 5 and M18 is pyoluteorin (plt). Some strains of *P. fluorescens* also produce a toxin with insecticidal activity (FitD-fluorescent insect toxin) which is closely related to *mcf* present in genome of *Pseudomonas chlororaphis* GP72 and is associated with lethality against tobacco hornworm. The genes for regulation and efflux of FitD are located on the *fitABCDEFGH* locus within the 90 gene insertion acquired by HGT (Pechy-Tarr et al. 2008). For the transport of extracellular enzymes, genomes of *P. fluorescens* have 1–3 T2SS for transport of lipases, esterases and alkaline phosphatases. Of the four different types of T2SS, three are related to Xcp and Hxc system of *P. aeruginosa*. Several of T3SS and T6SS for the delivery of effector molecule have also been identified in *P. fluorescens* genome.

4.3.7 *Pseudomonas stutzeri*

P. stutzeri A1501 is capable of fixing atmospheric nitrogen and thus results in direct plant growth promotion. The genome of *A. stutzeri* A1501 contains a cluster of 59 genes specific for nitrogen fixation and the *nif* operon shows a high degree of similarity to that in the genome of *Azotobacter vinelandii*. Genomes of GP72, Pf 5 and M18 contain 13, 13 and 14, respectively, homologous genes with A1501 and however lacked the nitrogenase complex-encoding genes *nif DK* (Roberts et al. 1978; Yan et al. 2010).

Besides *P. stutzeri* A1501 genome sequences of 10 other strains including one clinical isolate CGMCC1.1803; one naphthalene-degrading strain CCUG29243; one carbazole degrader, strain XLDN-R; one arsenite-oxidizing strain (TS44); model strains for denitrification CCUG16156; T13; a natural transformation strain DSM10701; lactate utilization strain SDM-LAC; and a nitrogen fixer strain DSM 4166 are already available at gene bank (Chen et al. 2011; Liu et al. 2012; Peña et al. 2012; Busquets et al. 2012; Jiang et al. 2012). The genome size of *P. stutzeri* generally varies from 4.17 to 4.70 Mb and is considerably smaller than the genomes other *Pseudomonas*. However, the draft genome of *P. stutzeri* strain B1SMNI has a size of 5.2 Mb, i.e. the highest described for *P. stutzeri* species; the strain also has two plasmids measuring 44,324 bp and 56,118 bp. GC ratio for the chromosome is 65.32 % and for plasmids 63.44 %. *Pseudomonas stutzeri* strain B1SMNI was isolated from waste water treatment plant and can grow in 2-methyl-naphthalene as a carbon and energy source and simultaneously fixed nitrogen under microaerophilic conditions. The total number of coding sequences is 4931 which code for metabolic and physiological functions including genes for starch

metabolism, flagellum synthesis and complete set of genes for nitrogen fixation as well as upper and lower operons for naphthalene and salicylate degradation. Sequences with properties of phages, transposons, integrons and insertion sequences were detected. Two plasmids, plasmid 1 and plasmid 2, code for 60 and 64 proteins, respectively, which carry functions such as conjugation and type IV secretion factors and are related to plasmid biology and transposases. Comparison with other complete genome sequences showed 96 % similarity within genomovars, 80–93 % for strains of different genomovars and lower of 77 % with other *Pseudomonas* spp. (Busquets et al. 2013).

Genome of *P. stutzeri* strain KOS6 isolated from industrial hydrocarbon sludge and capable of fixing nitrogen has a genome size of 5,014,616 with G+C ratio of 62.55 %. Besides the housekeeping genes and identification system contain genes which are important for phytoremediation technology. These genes are involved in plant growth promotion, resistance to toxic compounds and aromatic and polycyclic aromatic hydrocarbon degradation (Brunet-Galmés et al. 2012; Grigoryeva et al. 2013).

Recently the draft genome sequence of *Pseudomonas bauzanensis* strain W13Z2 has been published (Wang et al. 2014). Strain W13Z2 is halotolerant (5 % NaCl) and capable of degrading polycyclic aromatic hydrocarbons phenanthrene and pyrene. The genome of *P. bauzanensis* strain W13Z2 has been reported to consist of 8.6 Mb the largest of all the sequenced genomes of *Pseudomonas* spp. with G+C ratio of 61.8 %. A total number of 7839 coding sequences (CDS), 139 pseudogenes, 97 tRNA genes and 2 noncoding RNA (ncRNA) and 11 rRNA genes were identified. Of the CDS, 76.5 % were assigned to clusters of orthologous groups (COG) with amino acid transport and metabolic pathways. Average nucleotide identity (ANI) analysis revealed that *P. bauzanensis* W13Z2 is phylogenetically related to *P. aeruginosa* PAO1 (70.5 %), *P. putida* KT2440 (69.8 %), *P. entomophila* L48 (70.2 %), *P. mendocina* NK01 (70.5 %), *P. stutzeri* A1501 (70.8 %) and *P. syringae* pv. tomato DC3000 (69.2 %) (Stover et al. 2000; Vodovar et al. 2006; Nelson et al. 2002; Yan et al. 2008; Buell et al. 2003).

4.4 Summary

Genome of *Pseudomonas* exhibits a combination of features of terrestrial, aquatic, rhizospheric bacteria as well as those of human, animal, plant and insect pathogens. Genus comprising of more than 200 species are highly versatile in metabolism and opportunistic proteobacteria that inhabit a wide range of environment. Such is the diversity in ecological habitat, metabolism and interaction with other biological systems that the extensive repertoire of functional traits is reflected in the larger sized genome as compared to the model bacterium, *E. coli*, and the potential biotechnological applications such as synthesis of chiral compounds required for synthesis of drugs, bioactive compounds, bioplastics as polymer of polyhydroxyalkanoates, biosurfactants and areas of bioremediation and biocontrol of plant

diseases and pests. *Pseudomonas putida* KT2440 is recognized as a model system for gene cloning and expression for synthesis of heterologous proteins.

Pseudomonas aeruginosa, the type species of the genus, infects patients with compromised immunity and is a cause of morbidity due to cystic fibrosis of lungs and nosocomial infections and shares 85 % of its CDS with nonpathogenic, metabolically robust environmental bacterium, *Pseudomonas putida*. *Pseudomonas putida* lacks genes for traits associated with pathogenicity except adhesion production but share traits of vast metabolic potential. *Pseudomonas fluorescens* genome represents only about 45–52 % as core genome compared to nearly 90 % of *P. aeruginosa*. Even within the species, different strains show 60 % similarity in proteins compared to 90 % among strains of *P. aeruginosa* and 70 % of *P. syringae*, thus indicating a greater diversity in *P. fluorescens*. The pan-genome of *P. fluorescens* shows 5798 CDS as having no orthologs in other species of *Pseudomonas* and these genes are probably acquired horizontally.

Further expansion of comparative genomic analysis, genome sequences, and build up of databases will go a long way to identify new genes, enzymes and pathways and help in establishing the role of the specific genes for in silico modelling and prediction of the potential biotechnological applications.

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Abha Shukla, Brijdeep Singh, Swaranjit Singh Cameotra,
and Rachhpal S. Kahlon

Contents

5.1	Introduction	194
5.2	Monooxygenases	196
5.2.1	Substrates for Monooxygenases	198
5.2.2	Cytochrome P450	204
5.3	Dioxygenases	205
5.3.1	Important Substrates for Dioxygenases	207
5.3.2	Phylogenetic Distribution and Diversity of Dioxygenase	210
5.3.3	Structure of Dioxygenases	212
5.4	Mechanism of Catalysis	218
5.4.1	Extradiol Dioxygenases	218
5.4.2	Intradiol Dioxygenases	220
5.4.3	Comparative Mechanism and Specificity of Dioxygenases	221
5.5	Applications of Oxygenases	222
5.6	Conclusion	222
	References	223

Abstract

Mono- and di-oxygenases are ubiquitous enzymes that catalyse the degradation of relatively unreactive and recalcitrant molecules by introducing one or two atoms of oxygen into the molecule. The ring structure is cleaved to give rise to

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linear molecules that are further metabolised. For their activity, they require reduction equivalents from NADH or NADPH and metal ions as coenzymes at the active site. These enzymes are considered important, and their applications may include production of chiral compounds for synthesis and fine chemicals for production of pharmaceuticals, biopolymers and for bioremediation and biosensors. As yet they are used as whole cells for some commercial processes as purified enzymes are highly specific for the substrate and requirements of coenzymes and cofactors. Controlling their activities and specificity is important for their rational functioning on large scale. Attempts are being made to engineer these enzymes by introducing modifications in the protein structure with a view to improve upon and introduce novel functions for these enzymes.

5.1 Introduction

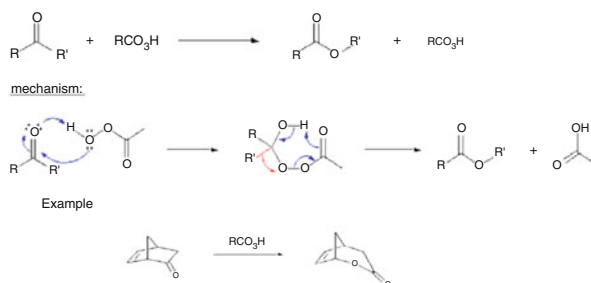
The biosphere has been formed by physical events and by interactions with the organisms that occupy it. Among the vast variety of living organisms, prokaryotes are metabolically much more diverse than eukaryotes and can flourish under a variety of extreme conditions where eukaryotes cannot. This is possible because of the wealth of various genes, metabolic pathways and molecular processes that are unique to prokaryotic cells; furthermore, they are more exposed to the environmental contaminants and are evolved in such a way that they successfully survive in those conditions. For this reason, prokaryotes are very important in the bioremediation of the environment contaminated with different toxic compounds like pesticides, herbicides, colouring dyes, petroleum hydrocarbons, etc., and they also play an important role in the cycling of elements, including carbon, nitrogen, sulphur and phosphorus, as well as metals and metalloids such as cobalt, chromium, copper, cadmium, arsenic, selenium, etc. (Andreazza et al. 2011; Dhanjal and Cameotra 2010; Fetzner 2012; Illanjiam and Arunachalam 2012; Karunya and Reetha 2012; Pratheesh and Jayachandran 2012).

There are a number of enzymes found in bacteria for its regular functions, and some enzymes are bacterial specific which contribute to some distinguished function in that organism. The enzymes found in the prokaryotes are of great potential, and with the aid of biotechnology these enzymes have found application in various production industries where these enzymes are used for catalysing various reactions to obtain the product of interest (Tang and Zhao 2009). Out of these enzymes, oxygenases are known to incorporate molecular oxygen directly into organic substrate with high selectivity. Various aromatic-oxidised compounds, dialcohols, dialdehydes, dicarboxylic acids and long-chain epoxides are prepared enzymatically by oxidising aromatic and aliphatic compounds with oxygenases (Joo et al. 1999). Hayaishi was the first to isolate and purify oxygenase and named it

‘pyrocatechase’ (Hayaishi and Hashimoto 1950; Hayaishi 1966). The enzyme was purified from soil bacterium, identified as *Pseudomonas* isolated by enrichment with tryptophan as sole source of carbon and nitrogen and catalyses the oxidative cleavage of catechol to cis-cis-muconate. *Pseudomonas* oxygenases are soluble and can be easily extracted from the bacterial cells. These oxygenases can be classified into two groups: (1) the monooxygenase, in this only one atom of oxygen is incorporated into the substrate molecule and the other atom is reduced to H₂O molecule by accepting hydrogen from the suitable electron donor like pyridine nucleotide, flavin nucleotide, ascorbic acid or reduced peridine nucleotide and (2) the dioxygenase, in which case two atoms of molecular oxygen are incorporated into the ring structured molecule forming a diol.

Among prokaryotes, the genus *Pseudomonas* comprises a vast group of bacteria that are found in a variety of natural environment such as soil, freshwater, and marine water and in many different associations with plants and animals. It belongs to the family Pseudomonadaceae containing over 200 validly published species. Their ecological diversity reflects their simple nutritional requirement, adaptation and ability to metabolise a large group of compounds. There has been a great interest among researchers in understanding various pathways and mechanisms used by this genus for the degradation of wide range of compounds. Metabolic pathways and encoding genes have been characterised in many strains of *Pseudomonas* and related genera. This Gram-negative genus has been extensively studied for its active involvement in pathogenesis, plant-bacterial interaction, various bio-transformations in production of products of interest and remediation processes and in biotechnological applications. The experimental work on the ability of this genus to utilise a wide variety of substrates including the highly stable planar aromatic compounds brought into light the concept of ‘metabolic plasmids’; for example, a 140 megadalton plasmid for camphor degradation has genes clustered in an operon which encode enzymes that initiate fission in the terpene ring through various processes followed by lactone formation involving Baeyer-Villiger chemistry (Fig. 5.1), finally leading to acetate and isobutyrate, which represents an interesting biotransformation (Gunsalus et al. 1975; Chakrabarty 1976).

Fig. 5.1 Mechanism of Baeyer Villiger rearrangement, works with H₂O₂ under basic conditions as well, the most electron-rich group migrates and the migrating group retains its stereochemistry



5.2 Monooxygenases

Monooxygenases, a family of enzymes of great interest for bioremediation of contaminated soil, are known to act as biocatalysts in bioremediation and synthetic chemical industry due to their high regio- and stereoselective nucleophilic and electrophilic bio-oxygenation of a wide range of substrates (Chauhan et al. 1998; Gallagher et al. 1997; Takahashi et al. 2007). Their subunit organisation and composition defines their function in terms of substrate specificity. The rearrangements of subunits as well as employments of new ones can be used to explain their different properties and functionalities. The observed pattern can be streamlined appealing to a number of evolutionary events, including horizontal gene transfer (Notomista et al. 2003). Their involvement in the processes like desulphurisation, denitrification, hydroxylation, dehalogenation, degradation and transformation of various compounds, e.g. biosynthesis of antibiotics and siderophores, etc., makes them significant and competent in modern chemical synthesis and environmental bioremediation.

Aerobic bacterial degradation of aromatic hydrocarbons is generally divided into two major routes, the so-called upper pathway, which leads to the formation of partially oxidised aromatic intermediates and a lower pathway, which uses dihydroxylated aromatic molecules. These activated aromatic compounds undergo ring-cleavage reactions and are further processed to give molecules that can eventually enter the citric acid cycle. In aerobic microorganisms, activation of an aromatic substrate is achieved through oxygenation reactions catalysed by Rieske non-haem iron oxygenases (Gibson and Parales 2000) or through soluble di-iron family of monooxygenases (Leahy et al. 2003), and the critical de-aromatisation step is performed by ring-cleaving dioxygenases. In *Pseudomonas* oxygenases are involved in activation of aromatic ring and involve the incorporation of oxygen atom(s) directly into the organic molecule. The oxygenase reactions are highly exergonic, and the free energy released is not conserved in the form of ATP. Monooxygenases are key enzymes in the upper pathway and catalyse hydroxylation of the aromatic ring at different positions.

So far two classes of flavoprotein hydroxylases have been shown to act as aromatic monooxygenases: the single-component aromatic hydroxylases (Suske et al. 1997) and two-component hydroxylases (Arunachalam et al. 1994; Becker et al. 1997). The single-component aromatic hydroxylases share a typical dinucleotide binding fold for complexation of the FAD cofactor while lacking a NAD (P) binding fold. The two-component hydroxylases typically consist of relatively small flavin reductases which generate reduced flavin at the expense of NAD(P)H. After intermolecular transfer, the reduced flavin is used by the large oxygenating component to catalyse substrate hydroxylation (Xun and Sandvik 2000). Further monooxygenases are classified into two subcategories based upon cofactor incorporated, i.e. flavin dependent which contain flavin as prosthetic group and require NADP or NADPH as coenzyme and P450 monooxygenases which are haem containing monooxygenases and can be found in prokaryotic as well as eukaryotic cells. Other types of bacterial monooxygenases, e.g. pterin-dependent

monooxygenases and metal ion-dependent monooxygenases, are also known to have ions such as copper, binuclear iron centres and mononuclear iron centres that do not contain flavin or haem (Arp et al. 2001). Commonly, monooxygenases require cofactor for their activities; however, some monooxygenases have been identified and characterised that do not require any cofactor for their activities. These cofactor-independent monooxygenases require only molecular oxygen for their activities and utilise the substrate as reducing agent, for example, tetracenomycin F1 monooxygenase (TcmH) from *Streptomyces glaucus* and quinol monooxygenase (YgiN) from *Escherichia coli*.

Oxygenases are widespread among the *Pseudomonas* species which are generally encoded by genes located on large catabolic plasmids such as CAM, TOL, XYL, NAH, OCT and SAL which initiates the first step in the oxidation of camphor, toluene, *p*- and *m*-xylene, naphthalene, *n*-alkanes and salicylate, respectively. Different types of monooxygenases are listed in Table 5.1.

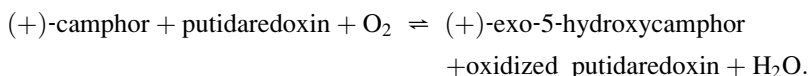
Table 5.1 List of important monooxygenases found in *Pseudomonas* species

S. No.	Enzyme	Substrate(s)	References
1.	Toluene 2-monooxygenase	2-Hydroxytoluene, toluene, 1,1,2-trichloroethylene	Olsen et al. (1994)
2.	Styrene monooxygenase	Styrene, indene, indole	Hartmans et al. (1990)
3.	Dibenzothiophene 5,5-dioxide monooxygenase	Dibenzothiophene-5,5-dioxide	Beil et al. (1996)
4.	Toluene sulfonate methyl monooxygenase	Toluene-4-sulfonate	Campos-Garcia et al. (1999)
5.	3-Hydroxybenzoate 4-hydroxylase	3-Hydroxybenzoate	Chapman and Ribbons (1976)
6.	3-Hydroxybenzoate 6-monooxygenase	3-Hydroxybenzoate	Nakazawa and Hayashi (1978)
7.	4-Methoxybenzoate monooxygenase	4-Methoxybenzoate	Poh and Bayly (1980)
8.	Benzoate 4-monooxygenase	Benzoate	Bernhardt et al. (1988)
9.	Butane monooxygenase	Butane	Sluis et al. (2002)
10.	DCA monooxygenase	1,2-Dichloroethane	Hage and Hartmans (1999)
11.	2,5-Diketocamphane 1,2-monooxygenase, camphor 5-monooxygenase	Camphor	Taylor and Trudgill (1986), Grogan et al. (1993)
12.	<i>p</i> -cymene monooxygenase	<i>p</i> -cymene, 4-chlorotoluene, 4-ethyltoluene and 4-methylthiotoluene	Nishio et al. (2001)
13.	4-Hydroxyacetophenone Monooxygenase	4-Hydroxyacetophenone and other aromatic ketones	Rehder et al. (2009), Kamerbeek et al. (2003)

5.2.1 Substrates for Monooxygenases

5.2.1.1 Camphor Monooxygenases

Camphor is a well-known bicyclic monoterpenoid having two enantiomeric forms, i.e. (+)-camphor and (–)-camphor, distributed in nature, e.g. in the wood of *Cinnanomum camphora* or in the essential oils from different plants, although (+) form is widely distributed in comparison to (–) form. *Pseudomonas putida* ATCC 17453 is well known to grow on both types of camphor enantiomers (LeGall et al. 1963). Many camphor monooxygenases are reported as camphor 5-monooxygenase, camphor 1,2-monooxygenase, camphor 1,6-monooxygenase, etc. Camphor 5-monooxygenase enzyme catalyses the chemical reaction:



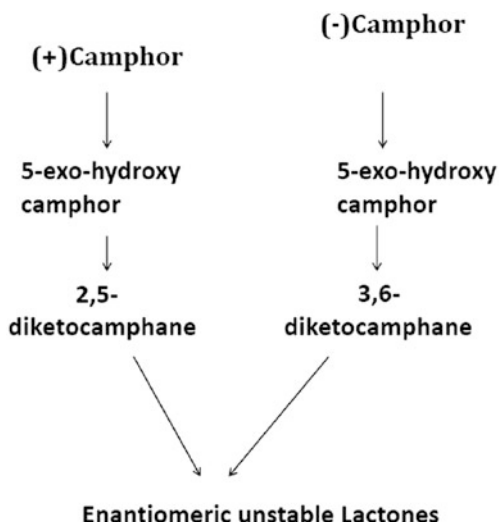
In the initial growth phase on camphor, a monooxygenase, which is a cytochrome-P450-containing enzyme complex, is induced and forms 5-exo-hydroxycamphor from (+)-camphor and hydroxylates (–)-camphor in the corresponding enantiomeric position (Gunsalus and Marshall 1971).

Camphor 1,2-monooxygenase which is a complex of an NADH dehydrogenase and a flavin mononucleotide containing oxygenating component catalyses the ring oxygenation of 2,5-diketocamphane formed from (+)-camphor and is specific for (+)-camphor and its derivatives and do not show the activity towards (–)-camphor and other ketocamphanes that do not possess a keto group at the 2-position. Jones et al. (1993) reported the purification of 3,6-diketocamphane 1,6 monooxygenase from (–)-camphor-grown *Pseudomonas putida* and 2,5-diketocamphane 1,2 monooxygenase from (+)-camphor *P. putida* ATCC17453. The study confirmed the enantiomeric specificity of the monooxygenase obtained from the growth of *P. putida* on (–)-camphor. The immunological investigation analyses of the two proteins revealed a close relation between them and were found almost identical in size and subunit structure. SDS-PAGE of 3,6-diketocamphane 1,6-monooxygenase revealed that it consists of two electrophoretically identical subunits which are about 3 kDa smaller than the pair from which the 2,5-diketocamphane monooxygenase is constructed. Both enzymes bind flavin in a non-covalent manner, and each apoenzyme is specifically reactivated by FMN, and both can make use of the NADH dehydrogenase induced by growth with (+)-camphor as the electron donor. Table 5.2 shows the specificity of the two enantiomeric enzymes for different substrates.

Although the specific activity of 3,6-diketocamphane monooxygenase was low for both the substrates, i.e. (+)-camphor and (–)-camphor, later was found to be a better inducer for 3,6-diketocamphane monooxygenase (Fig. 5.2).

Table 5.2 Comparative data of the activity of both the purified enzymes against different substrates (Jones et al. 1993)

Growth substrate	Enzyme	Total activity (U)
(+)-Camphor	2,5-diketocamphane monooxygenase	29.6
(+)-Camphor	3,6-diketocamphane monooxygenase	8.4
(-)-Camphor	2,5-diketocamphane monooxygenase	54.3
(-)-Camphor	3,6-diketocamphane monooxygenase	15.9

Fig. 5.2 Early reactions in the degradation of camphor (Jones et al. 1993)

5.2.1.2 Toluene/*o*-Xylene Monooxygenase

Toluene monooxygenases, which include monooxygenases from both the TMO and phenol hydroxylase (PH) subclasses, have evolved to hydroxylate toluene specifically at the *ortho*-, *meta*- and *para*-positions, with high regiospecificity, and to epoxidise alkenes with high stereospecificity. Two different monooxygenases have been found in the genome of *Pseudomonas stutzeri* OX1, phenol hydroxylase (PH) and toluene/*o*-xylene monooxygenase (ToMO). The toluene/*o*-xylene monooxygenase (ToMO) is a four-component monooxygenase purified from *Pseudomonas stutzeri* OX1 and have relaxed toluene specificity (Bertoni et al. 1996), yet produce highly regiospecific products from alternate substrates like *o*-xylene, dimethylphenol and *m*-cresol and is capable of oxidising arenes, alkenes and halo alkanes at a carboxylate-bridged di-iron centre. Due to the ability of oxidising a wide range of substrates, the enzyme invites applications ranging from bioremediation to the regio- and enantio-specific oxidation of hydrocarbons on an industrial scale. Substrate hydroxylation in BMMs occurs at a dioxygen-activated, carboxylate-bridged di-iron centre in the α -subunit of a ~220–250 kDa hydroxylase component that is an $(\alpha\beta\gamma)_2$ heterodimer or, in the case of one known AMO, an $\alpha\beta$ monomer (Rosenzweig et al. 1993; Miura and Dalton 1995; Leahy et al. 2003).

The crystal structure of the toluene/*o*-xylene monooxygenase hydroxylases was very well characterised by Sazinsky and coworkers (2004) which offers an insight into the different substrate specificities of multicomponent monooxygenases and explains the behaviour of mutant forms of homologous enzymes described in the literature.

On the other hand, phenol hydroxylase (PH) is a multicomponent protein belonging to group 1 BMMs that includes well-characterised enzymes such as PH from *Pseudomonas* sp. strain CF600 and T2MO from *B. cepacia* G4 and is more closely related to the *Pseudomonas* sp. strain CF600 enzyme. Subunits L, N and O of *P. stutzeri* OX1 PH constitute a dimeric (LNO)₂ subcomplex with the presence of a di-iron centre in each of the N subunits. Phenol hydroxylases (PH) (LNO)₂ alone, in the absence of any other PH component, are able to convert phenol into catechol. PH P homology studies have shown that this subunit is homologous to the reductase components of other BMMs. The spectrum of purified PH P revealed the presence of a [2Fe–2S] iron-sulphur cluster and a flavin cofactor. PH P can reduce several artificial electron acceptors with either NADH or, less efficiently, NADPH as the electron donor. These observations lead to the identification of subunit PH P with the NAD(P)H-oxidoreductase component of the PH complex. PH P is not able to convert phenol into catechol in single-turnover experiments, but its addition to the hydroxylase subcomplex (LNO)₂ increased the amount of the product produced 2.48 folds. Cafaro et al. (2004) examined the interplay between the two enzymes, i.e. toluene/*o*-xylene monooxygenase and phenol hydroxylase. They reported that when both enzymes compete for benzene as a substrate, ToMO will efficiently produce phenol, which in turn will be very efficiently oxidised to catechol by PH. Cafaro et al. (2005) investigated the kinetics and regioselectivity of toluene and *o*-xylene oxidation using *E. coli* cells expressing ToMO and PH complexes and found that in the recombinant system, the enzymes act sequentially and that their catalytic efficiency and regioselectivity optimise the degradation of toluene and *o*-xylene. The main product of toluene oxidation by ToMO is *p*-cresol, which is a best substrate for PH, which catalyses its transformation to 4-methylcatechol. The sequential action of the two enzymes on *o*-xylene leads, via the intermediate 3,4-dimethylphenol, to the restricted production of 3,4-dimethylcatechol, the only dimethylcatechol isomer that can serve as a carbon and energy source after further metabolic processing. The study also gives a metabolic explanation for the acquisition of the ToMO operon by *P. stutzeri* OX1. It is possible that using the two enzymes in a concerted fashion confers on the strain a selective advantage based on the ability of the microorganism to optimise the efficiency of the use of nonhydroxylated aromatic hydrocarbons, such as benzene, toluene and *o*-xylene.

5.2.1.3 *p*-Cymene Monooxygenase

The metabolism of *p*-cymene (known as cym pathway) is initiated via monooxygenation by *p*-cymene monooxygenase, a two-component enzyme which, in the presence of NADH, effects the insertion of one atom of activated molecular oxygen into the methyl group, resulting in *p*-isopropylbenzyl alcohol (*p*-cumin alcohol).

Subsequent reactions lead to the formation of *p*-cumate (*p*-isopropylbenzoic acid) which is metabolised to isobutyrate, pyruvate and acetyl-CoA. Nishio et al. (2001) studied the biotransformation of a number of substrates by cloned *p*-cymene monooxygenase isolated from *Pseudomonas putida* F1. *P*-Cymene monooxygenase (CMO) consists of a hydroxylase (CymA1) and a reductase component (CymA2) which initiate *p*-cymene (*p*-isopropyltoluene) catabolism by oxidation of the methyl group. The amino acid sequence of CymA1 and CymA2 is related to the XylM and XylA components, respectively, of the XMO of *P. putida* mt-2 (Suzuki et al. 1991). They studied the diverse range of substrates catalysed by CMO, by cloning the *cymA1A2* genes in an *E. coli* pT7-5 expression system, and these cells were used in transformation experiments. The tested substrates include different substituents on the aromatic ring at the 2 (*ortho*), 3 (*meta*) or 4 (*para*) position relative to the methyl moiety. As a result, a distinct preference was observed for substrates containing at least an alkyl or heteroatom substituent at the *para*-position of toluene. Besides *p*-cymene, the substrate range of CMO of *PpF1* was found to include 4-chlorotoluene, 4-ethyltoluene and 4-methylthiotoluene. Unlike XMO, CMO is not able to metabolise toluene [which is the function of a dedicated *tod* pathway (Gibson et al. 1990)].

5.2.1.4 Soluble Butane Monooxygenase

Growth on C2–C4 gaseous alkanes is largely attributed to the *Corynebacterium-Nocardia-Mycobacterium-Rhodococcus* complex of Gram-positive bacteria (Ashraf et al. 1994; McLee et al. 1972; Perry 1980). An exception to this association is the Gram-negative *Pseudomonas butanovora* (ATCC 43655). Soluble butane monooxygenase (sBMO) from *Pseudomonas butanovora* is a multimeric hydroxylase which catalyse the oxidation of butane to 1-butanol. The butane monooxygenase of *P. butanovora* has also been implicated in the co-oxidation of chlorinated organic compounds and thus has potential use for bioremediation. Sluis et al. (2002) cloned six structural genes *bmoXYZZDC* identified for partially purified sBMO from *P. butanovora* and sequenced.

The study suggested that it is a multicomponent enzyme composed of hydroxylase, regulatory protein and a pyridine nucleotide (e.g. NADH) oxidoreductase, i.e. a three-component di-iron monooxygenase system (Dubbels et al. 2007). In their study, they found that the sBMO complex was composed of an iron-containing hydroxylase (BMOH), a flavo-iron sulphur-containing NADH-oxidoreductase (BMOR) and a small regulatory component protein (BMOB). The oxidoreductase component of sBMO exhibited molecular masses of 45 kDa by denaturing SDS-PAGE, as reported by Sluis et al. (2002) and 40.2 kDa by analytical ultracentrifugation. As hypothesised by Sluis et al. (2002), that there might be a third component incorporating with BMOH and BMOR, Dubbels and coworkers detected the presence of BMOB. In their work, BMOB was found to co-elute with BMOH and could be separated by including 1 M KCl during the gel-filtration step of the purification. The identity of BMOB was verified by

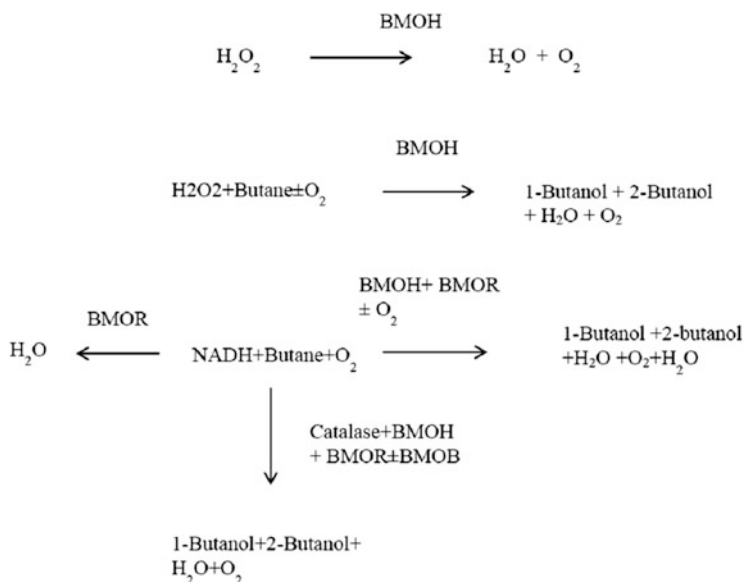


Fig. 5.3 The reactions of soluble butane monooxygenase components (Dubbels et al. 2007)

Edman degradation analysis. Denaturing SDS-PAGE of BMOB revealed an apparent molecular mass of 22 kDa, an unexpected result considering the predicted mass of 15.1 kDa from the deduced amino acid sequence (Sluis et al. 2002). Gel filtration studies of BMOH suggested its molecular masses between 200 and 260 kDa, and denaturing SDS-PAGE revealed that it consisted of three proteins in stoichiometric amounts exhibiting apparent molecular masses of 54, 43 and 25 kDa as previously observed (Sluis et al. 2002).

These results suggest that, like other multicomponent monooxygenase systems, the holoenzyme has a $(\alpha\beta\gamma)_2$ quaternary structure and is consistent with the deduced amino acid sequences of the three subunits of the butane monooxygenase operon. The studies on catalytic property of the system showed that the hydroxylation of the substrates coupled with NADH oxidation requires only two components, i.e. BMOH and BMOR, while the addition of BMOB to the system slightly increased the activity of the system. Preferential production of primary alcohols required for downstream catabolism of *P. butanovora* was found to be independent of the small component protein, BMOB (Fig. 5.3).

5.2.1.5 4-Hydroxyacetophenone Monooxygenase

4-hydroxyacetophenone monooxygenase (HAPMO), a Bayer-Villiger monooxygenase, belongs to family oxidoreductase containing flavin and is known to transform aromatic ketones into corresponding aromatic esters or lactones. The

enzyme is reported to be purified from *Pseudomonas fluorescens* ACB and *Pseudomonas putida* JD1 (Kamerbeek et al. 2003; Rehdorf et al. 2009). HAPMO utilises a wide range of substrates of the aromatic/cyclic ketones family. Characterisation of the purified enzyme showed that 4-hydroxyacetophenone monooxygenase is a homodimer of <140 kDa with each subunit containing a noncovalently bound FAD molecule (Kamerbeek et al. 2001). The sequence of HAPMO shares significant similarity with two known types of Baeyer-Villiger monooxygenases: cyclohexanone monooxygenase (27–33 % sequence identity) and steroid monooxygenase (33 % sequence identity).

Kamerbeek et al. (2001) reported the purification and characterisation of HAPMO from *Pseudomonas fluorescens* ACB. The catalyses of HAPMO was found to be FAD dependent during conversion of 4-hydroxyacetophenone to 4-hydroxyphenyl acetate with consumption of stoichiometric amounts of NADPH and molecular oxygen. The enzyme was found to show maximum activity at pH 7.5/8.0 and temperature 30 °C. The specific activity of the purified enzyme was found to be 5.5 mmol NADPH oxidised min⁻¹ mg⁻¹ under standard assay conditions. Addition of free FAD to the assay mixture did not stimulate HAPMO activity, indicating that the flavin cofactor is tightly bound. The highest catalytic efficiency of the enzyme was observed with compounds bearing an electron-donating substituent at the para-position of the aromatic ring while HAPMO was not able to convert cyclohexanone and cyclopentanone, which are substrates for some known Baeyer-Villiger monooxygenases (Mihovilovic et al. 2006). HAPMO activity in crude extracts of *P. putida* JD1 with number of compounds showed that the enzyme is active with all the three classes, i.e. aromatic, heteroaromatic and aliphatic compounds (Rehdorf et al. 2009). Furthermore, they cloned, expressed and characterised 4-hydroxyacetophenone monooxygenase from *Pseudomonas putida* JD1 in *E. coli*. The biocatalytic investigation of the enzyme revealed that it is active for a wide range of arylaliphatic ketones with preferences for para substitutions in the aromatic ring. Table 5.3 represents the list of compounds which were transformed by HAPMO.

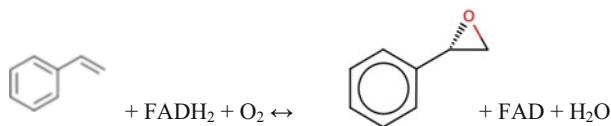
Table 5.3 Biocatalysis of β -hydroxy-2-ketones using crude extract (*E. coli* Rosetta pET22b(+)-PpDJ1HAPMO)

Substrate	Time (h)	ee _p (%)	Conversion (%)	<i>E</i>	Product
4-Hydroxy-2-octanone	4	12.4	2.4	1.3	4-Hydroxyhexylacetate
4-Hydroxy-2-nonanone	4	24.0	4.8	1.6	4-Hydroxyheptylacetate
4-Hydroxy-2-decanone	4	50.2	3.0	3.0	4-Hydroxyoctylacetate
4-Hydroxy-2-undecanone	4	6.4	8.6	1.1	4-Hydroxynonylacetate

Rehdorf et al. (2009). ee_p—Enantiometric excess of the product; the conversion an *E* value

5.2.1.6 Styrene Monooxygenase

Styrene monooxygenase is an enzyme that catalyses the chemical reaction.



Styrene oxide formed is then transformed into phenylacetaldehyde in the presence of styrene oxide isomerase (Hartmans et al. 1990), which then further degrades and enters into TCA cycle depending upon the enzymes present in the organism. Although some other substrates like indene, indole, etc., are also transformed by this enzyme (Gursky et al. 2010). This is a two-component flavin enzyme, belongs to the group oxidoreductase and depends on FAD as a cofactor (Kantz et al. 2005). So far two types of styrene monooxygenase are reported in literature: StyA/StyB (designated E1), first described from *Pseudomonas* species (Kantz et al. 2005), and StyA1/StyA2B (designated E2), described from Actinobacteria (Tischler et al. 2010). The former, i.e. E1, is more abundant in nature and comprises a single monooxygenase (StyA) supported by a single reductase (StyB), whereas the E2-type has a major monooxygenase (StyA1) which is supported by fusion protein of a monooxygenase and reductase (StyA2B). To date, all styrene monooxygenases perform enantioselective epoxidations of styrene and chemically analogous compounds, which makes them interesting for industrial applications. Gursky et al. (2010) conducted in vitro evolution in *styAB* genes from *Pseudomonas putida* CA-3, which encode styrene monooxygenase using error-prone polymerase chain reaction. Libraries of the variants were generated after each round of evolution, and variants were screened for the improved conversion of indene to indigo. The mutation caused in the genes after three rounds of evolution increased the efficiency of the variant cells to transform styrene and indene from eight- to twelvefolds in comparison to wild-type strain with excellent enantioselectivity. This type of studies can generate efficient biocatalysts and can motivate the use of these enzymes on industrial scale.

5.2.2 Cytochrome P450

Cytochrome P450 are regio- and stereospecific haem proteins belonging to external monooxygenases which are capable of insertion of oxygen into almost all class of molecules, and so they are able to transform a number of compounds. Therefore, this biocatalyst has a great potential in drug industry, targeted cancer gene therapy, biosensor design, synthesis of various chemicals and in bioremediation. They receive the necessary electrons for oxygen cleavage and substrate hydroxylation from different redox partners. So far different types or classes of cytochrome P450 are known (Hannemann et al. 2007). Smith et al. (2004) reported the involvement of

newly identified cytochrome P450 in the degradation of dihydroabietic acid and palustric acid found in *Pseudomonas abietaniphila* BKME-9. Many researchers have studied the catalytic pathway and crystal structures of cytochrome P450_{cam} from *Pseudomonas putida* (Poulos et al. 1985; Schlichting et al. 2000). Using reported structure studies of cytochrome P450_{cam} from *Pseudomonas putida*, Poulos and Raag (1992) reviewed in detail how cytochrome P450 functions, changes in its absorption spectra in different conditions and affecting factors such as substrate-free and substrate-bound conditions, structure of carbon monoxide complex, etc., Guengerich (1995) have described the potential utilisation of cytochrome P450 proteins in biodegradation. However, there are still some questions which are not very clear regarding the mechanism of catalysis. However, the use of isolated P450 enzymes in biocatalysis is challenging as they are intrinsically not very active and have poor stability in isolated form. Membrane-bound proteins such as ferredoxin and FMN reductases are used for electron transfer. Furthermore, NAD (P)H which is a very expensive chemical is consumed during the reaction; therefore, a process is required to regenerate it or it should be replaced by some other cofactors. In spite of these obstacles, this group of enzymes is attracting a large number of biotechnologists to explore the ability of this wonder enzyme in different fields and to make it competent on industrial scale (Kumar 2010).

5.3 Dioxygenases

The dioxygenases are a group of enzymes that are involved at several stages of catabolic pathways of aromatic compounds. In contrast to monooxygenases, these incorporate two atoms of oxygen per molecule of the aromatic substrate, giving rise to catechols or allied compounds which are further metabolised, and the products formed enter the central metabolic pathway.

Pseudomonas oxygenases have been extensively studied, and besides their significance in understanding the metabolism of aromatic compounds, the ring-cleavage dioxygenases are of interest for development of suitable strains for the degradation of chemical pollutants such as polychlorinated biphenyls (PCBs), benzenes and other industrial chemical solvents and pollutants (Bugg and Ramaswamy 2008; Vaillancourt et al. 2006). These organisms hold great potential for degradation of chemical pollutants and bioremediation applications.

Pseudomonads use different pathways for degradation of each type of aromatic compound, and the pathway proceeds via one of the four intermediates, viz. catechol, protocatechuate, gentisate or hydroxyquinol (Fig. 5.4). Some other compounds such as homoprotocatechuate, dihydroxyphenyl propionate and gentisate also occur as intermediates. Salicylate and 2-aminophenol are also subjected to ring-cleavage reactions. Multiple benzene ring structure compounds are also degraded in the same manner as monocyclic compounds.

Dioxygenases are the key enzymes for cleavage of the aromatic ring by a two-step mechanism, (1) activation of the aromatic ring by introduction of substituents and (2) the dearomatisation. They use non-haem iron for the cleavage

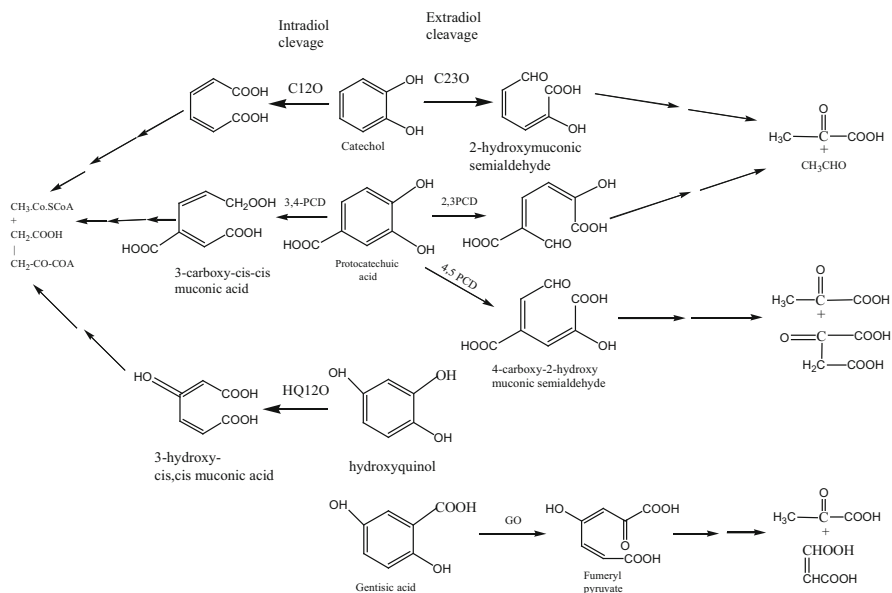


Fig. 5.4 Products of ring-cleavage dioxygenase-catalysed reactions. Intradiol and extradiol cleavage reactions. C12O–Catechol 1,2 dioxygenase; C23O–Catechol 2,3 dioxygenase; 3,4-PCD–3,4-Protocatechuric acid dioxygenase; 4,5-PCD–4,5-Protocatechuric acid dioxygenase; GO–Gentisic acid dioxygenase; HQ1,2O–Hydroxyquinol dioxygenase

reaction. Depending upon cleavage reaction, dioxygenases are grouped into two subclasses, the intradiol dioxygenases and the extradiol dioxygenases. The former utilise a non-haem mononuclear Fe(III) as cofactor, ligated by two tyrosine and two histidine ligands, to cleave aromatic ring in ortho, i.e. between the two hydroxyl group substituents. In contrast to this, the extradiol dioxygenases use non-haem mononuclear Fe(II) iron moiety as a cofactor, ligated by two histidine and one glutamate ligand and cleave the ring in the meta- position, i.e. adjacent to the hydroxyl group substituents. Some Mn²⁺- and Mg²⁺-dependent extradiol oxygenases bearing strong similarity to Fe⁺² enzymes have also been reported (Bugg and Ramaswamy 2008). The two types of dioxygenases have completely different structures and catalytic mechanisms (Fig. 5.5).

In the intradiol dioxygenases family which use a mononuclear Fe(III) centre, coordinated by two tyrosine and two histidine ligands, protocatechuate 3,4-dioxygenase is the most extensively studied enzyme. They cleave substrates possessing vicinal hydroxyl groups, e.g. catechol, protocatechuate and 2-hydroxyquinol (1,2,4-trihydroxybenzene), in the ortho position, i.e. between the two vicinal hydroxyl groups. In contrast to this, the substrates for extradiol dioxygenases need not possess vicinal hydroxyl groups and cleave bond in the meta position, i.e. adjacent to the hydroxyl group. Some of the noncatecholic compounds such as gentisate, hydroquinone, salicylate and 2-aminophenol are substrates for extradiol dioxygenases. They use mononuclear Fe(II) cofactor or rarely Mn²⁺ for catalysis. The types of reactions catalysed by this superfamily may

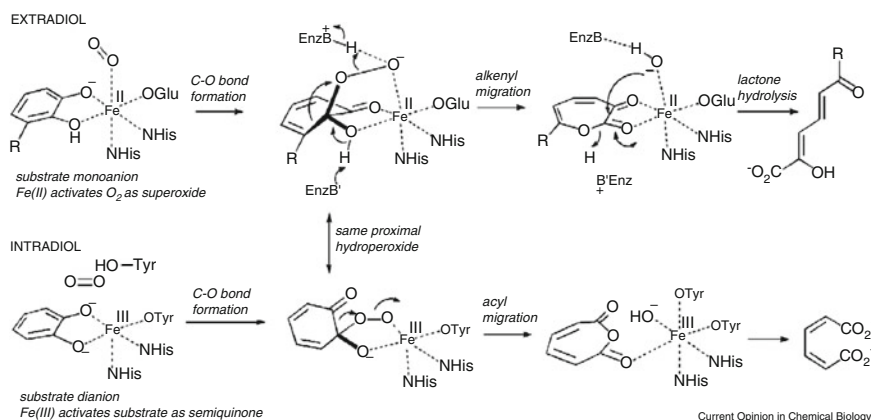


Fig. 5.5 Comparison of the catalytic mechanisms of the extradiol and intradiol catechol dioxygenases, showing the principal differences in catalytic strategy and alkenyl vs. acyl migration (with permission Bugg and Ramaswamy 2008)

include isomerisation, epimerisation, nucleophilic substitution and oxidative C–C bond cleavage (Bugg 2003).

Another difference between the two types of dioxygenases is that intradiol generally cleaves catechols possessing electron-withdrawing substituents, while the extradiol dioxygenases cleave catechols possessing electron-donating substituents. The dioxygenases are thus key enzymes in the degradation of aromatic compounds, and many of such proteins and their encoding sequences have been described, purified and characterised in the last decades. The extradiol dioxygenases are categorised into three different types, belonging to different families. Type I enzymes belong to the vicinal oxygen chelate family (Eltis and Bolin 1996), type II enzymes are related to LigB protocatechuate 4,5-dioxygenase (Sugimoto et al. 1999) and type III enzymes belong to the cupin superfamily (Dunwell et al. 2000). Importantly, new extradiol dioxygenases with novel properties are constantly being reported (Nogales et al. 2005; Moonen et al. 2008).

5.3.1 Important Substrates for Dioxygenases

5.3.1.1 Catechol

Catechol 2,3-dioxygenase (C23O) or meta-pyrocatechase was the first identified extradiol dioxygenase from *Pseudomonas*. The enzyme requires ferrous ion. Similarly, the first identified intradiol dioxygenase was catechol 1,2 dioxygenase (C12O) involved in catabolism of benzoates and require ferric iron. Catechol is the key intermediate formed in the degradation of benzene, benzoate, phenol and their alkylated, nitrosylated and halogenated derivatives (Hughes and Bayly 1983). Polycyclic aromatics give rise to substituted intermediates and may be considered as substituted catechols and are subjected to extradiol cleavage. Diterpenoids and steroids of plant origin are also degraded through formation of catechols.

Multiplicity of dioxygenases has been reported in *Rhodococcus* species, and strain RHA1 has been reported to degrade steroids through four different pathways (McLeod et al. 2006).

5.3.1.2 Protocatechuates

Protocatechuate are formed in the degradation of hydroxybenzoates, phthalates and vanillyl alcohols. Protocatechuate is subject to three different modes of cleavage: protocatechuate 3,4-dioxygenase (3,4-PCD) catalyses intradiol cleavage (Stanier and Ingraham 1954), whereas protocatechuate 2,3-dioxygenase (2,3-PCD) (Wolgel and Lipscomb 1990) and protocatechuate 4,5-dioxygenase (4,5-PCD) (Ono et al. 1970) catalyse each of the two different modes of extradiol cleavage.

The related compound, homoprotocatechuate (3,4-dihydroxyphenylacetate), is cleaved in an extradiol fashion by homoprotocatechuate 2,3-dioxygenase (HPCD) in the degradation of 4-chlorophenylacetate and 3- and 4-hydroxyphenylacetate. Related HPCDs utilising Fe^{2+} and Mn^{2+} have been isolated from *Brevibacterium fuscum* and *Arthrobacter globiformis* CM-2, respectively. A HPCD that utilises Mg (II) has also been purified (Wang and Lipscomb 1997; Whiting et al. 1996).

5.3.1.3 Gentisates and Salicylates

The ring cleavage of gentisate is catalysed by gentisate 1,2-dioxygenase (GO), an extradiol-type dioxygenase (Harpel and Lipscomb 1990). Gentisate has been identified as an intermediate in the catabolism of salicylate, 3-hydroxybenzoic acid and anthranilic acid. Homogentisate an important derivative of gentisate also occurs in the catabolism of aromatic amino acids, phenylalanine and tyrosine (Iwabuchi and Harayama 1998). GO has also been described that cleave salicylate (Hintner et al. 2001).

Related compounds that are subjected to extradiol-type cleavage include 5-aminosalicylate and 1-hydroxy-2-naphthoate which is involved in the catabolism of phenanthrene. 1-Hydroxy-2-naphthoate dioxygenase did not cleave salicylate or gentisate (Stolz et al. 1992).

5.3.1.4 Hydroxyquinols and Hydroquinones

Specific dioxygenases have been identified for catabolic cleavage of hydroxyquinols and hydroquinones. 2-Hydroxyquinols and its chlorinated derivatives are cleaved by hydroxyquinol 1,2 dioxygenase (HQ12O), an intradiol enzyme. This enzyme is important in the catabolism of *p*-nitrophenol, resorcinol and 4-hydroxysalicylic acid. The related chlorinated derivatives, 2,6-dichlorophenol and 2,4,6-trichlorophenol, are subject to catabolism by 6-chloro-hydroxyquinol 1,2-dioxygenase (6-chloro-HQ12O) (Zaborina et al. 1995). It is not clear whether HQ12O or 6-ClHQ12O is involved in the catabolism of 2-chlorophenol and 2,4-dichlorophenol. The hydroquinones are cleaved by an extradiol-type enzyme, hydroquinone dioxygenase (HQO). This enzyme is involved in the degradation of *p*-nitrophenol, pentachlorophenol (Xu et al. 1999) and lindane (Miyauchi et al. 1999).

5.3.1.5 2-Aminophenols

2-Aminophenols are cleaved by Fe(II)-dependent extradiol-type dioxygenases. The 2-aminophenol-1,6-dioxygenases (APDs) are involved in the degradation of nitrobenzene (Lendenmann and Spain 1996) and 2-aminophenol (Takenaka et al. 1997). 3-hydroxyanthranilate, an important derivative of 2-aminophenol, is an intermediate in the catabolism of 2-nitrobenzoate and tryptophan by *Pseudomonas* (Muraki et al. 2003).

5.3.1.6 Aromatic Amino Acids

Aerobic catabolism of aromatic amino acids by *Pseudomonas* also involves ring-cleavage mechanism. Thus, homogentisate dioxygenase (HGO) is involved in the catabolism of phenylalanine, and enzyme 3-hydroxyanthranilate dioxygenase (HAD) is involved in the catabolism of tryptophan by the kynurenine pathway. These dioxygenases, which are extradiol-type enzymes, occur in aerobic organisms ranging from *Pseudomonas* to man. In humans, HGO and HAD are associated with the genetic disorders alkaptonuria and Huntington's disease, respectively (Schwarcz et al. 1988).

5.3.1.7 Flavonols

Among the important flavonols are polyphenolic compounds synthesised by higher plants. Some examples are quercetin (3,5,7,3',4'-pentahydroxyflavone), kaempferol (3,5,7,4'-tetrahydroxy-flavone), myricetin (3,5,7,3',4',5'-hexahydroxyflavone) and isorhamnetin (3,5,7,4'-tetrahydroxy-3'-methoxyflavone). Quercetin is an antioxidant and has anti-inflammatory, antibacterial and anticancer properties (Cushnie and Lamb 2005; Erlund 2004; Pietta 2000). The initial step for aerobic metabolism of quercetin involves 2,4-dioxygenic ring cleavage by quercetinase to form 2-protocatechuoylphloroglucinol carboxylic acid and carbon monoxide.

Apart from their role in catabolic pathways, extradiol dioxygenases play a role in biosynthetic pathways of biologically active compounds (Novotna et al. 2004).

5.3.1.8 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAH) are added to the environment and are an important source of pollutants generated by oil and chemical industry in the environment. Microorganisms have evolved diverse catabolic pathways and enzyme system and play an indispensable role in the degradation and detoxification of PAH such as naphthalene, biphenyl, phenanthrene, anthracene, cumene, cymene, etc. (Boyd et al. 2001; Butler and Mason 1997). The initial step of aerobic degradation of PAH is an oxidative attack by insertion of molecular oxygen into the benzene ring catalysed by a dioxygenase (Fig. 5.6). Depending on the mode of hydroxylation, the substrate may be channelled into a productive route resulting in mineralisation, or the substrate may be co-metabolised leading to dead end product formation or intermediates that may further be catabolised by other bacteria in the population. The dioxygenases utilise non-haem iron (Fe^{2+}) and are referred to as Rieske-type non-haem iron aromatic ring-hydroxylating oxygenase (RHO).

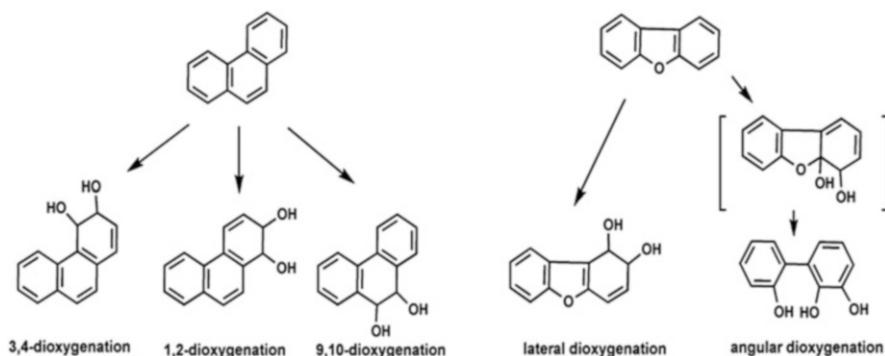


Fig. 5.6 Different regioselectivities of dioxygenolytic attack on polycyclic aromatic compounds, phenanthrene (*left*) and dibenzofuran (*angular dioxygenation*) (Vilchez-Vargas et al. 2010)

Although they utilise NAD(P)H as an electron donor and catalyse the same oxygenation reaction, they differ markedly in their structure. RHOs are multicomponent enzymes of two or three proteins comprising of an electron transport chain (ETC) and an oxygenase. Oxygenases are either homomultimers (α_n) or heteromultimers ($\alpha_n\beta_n$). The α subunit is larger and has two conserved regions, Rieske [2Fe–2S] centre and a non-haem mononuclear iron. The α unit is catalytic and transfers the electrons to oxygen molecules (Mason and Cammack 1992; Butler and Mason 1997). On the basis of the comparative study of 130 RHO enzymes and the sequence of genes encoding these, Kweon et al. (2008) proposed their classification into five groups (Type I–V). Type I and type III are further divided into subtypes $\alpha\beta$ and α depending on whether they are hetero-oligomers ($\alpha_n\beta_n$) or homo-oligomers (α_n). Along with the structure of the oxygenase, the reductant, used for transfer of electron, forms an important criterion for this classification. Some of the important products formed from respective substrates are naphthalene dihydrodiol, biphenyl dihydrodiol, phenanthrene 1 and phenanthrene 2, anthracene dihydrodiol, propylbenzene dihydrodiol, cumene dihydrodiol, etc. (Khara et al. 2013).

5.3.2 Phylogenetic Distribution and Diversity of Dioxygenase

Catabolic genes coding for extradiol dioxygenase activity show broad diversity and distribution. Important groups among these are represented by catechol 2,3-dioxygenases (Eltis and Bolin 1996) and LigB-type 2,3-dihydroxyphenylpropionate dioxygenases (Diaz et al. 2001) and TodE-like extradiol dioxygenases, belonging to subfamily I.3.B (Beil et al. 1999). High extradiol dioxygenase density in the polluted environment indicates that this function is responsible for biological fitness of the microbial community for bioremediation. Microbial community comprising of at least members of 14 bacterial orders (Kabelitz et al. 2009) has been reported under such conditions, and the encoding genes might be concentrated in multiple copies in specific bacterial hosts. Characterisation of gene

polymorphisms and predominant enzyme activities is important in identifying the catabolic potential of the microbial communities in contaminated sites. The *xylE* gene encoding catechol 2,3-dioxygenase of *P. putida* mt2 as well as related enzymes and their encoding genes are used as a marker for extradiol dioxygenase activity and characterisation of the catabolic potential of contaminated sites (Margesin et al. 2003). In fact, respective genes have been reported in various catabolic operons and have recently also been observed as being predominant at a site mainly contaminated with benzene (Junca et al. 2004).

Intradiol and extradiol enzymes do not share significant sequence or structural similarities and thus belong to evolutionary distinct classes of proteins. Sequence and structural analyses have shown that all intradiol dioxygenases characterised to date belong to a single evolutionary lineage. Thus, despite their different subunit compositions, the catalytic domains of 3,4-PCD and C12O share a common structural fold. Moreover, these enzymes share key conserved residues with the HQ12O including the four endogenous iron ligands (Broderick 1999; Bugg and Ramaswamy 2008). Genomic and metagenomic studies provided an insight into their phylogenetic distribution, diversity and abundance in the environment (Brennerova et al. 2009; Vilchez-Vargas et al. 2010).

In contrast to the intradiol dioxygenases, extradiol and extradiol-type dioxygenases belong to at least three evolutionarily independent families. The type I extradiol dioxygenases belong to the vicinal oxygen chelate (VOC) superfamily. Type I extradiol dioxygenase are of two different types depending upon their domains, the two-domain and one-domain enzymes. The typical examples of the two-domain type I extradiol dioxygenases are 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHBD) from *Burkholderia* sp. LB400 (DHBD_{LB400}), catechol 2,3 dioxygenase from *P. putida* mt-2(C23O_{mt2}), homoprotocatechuate 2,3-dioxygenase from *Brevibacterium fuscum* (HPCD_{Bfu}) and chlorohydroquinone dioxygenase from *R. globerulus* P6 (CHQO) (Xu et al. 1999; Vetting et al. 2004). The one-domain type I extradiol dioxygenases are 2,3-dihydroxybiphenyl 1,2-dioxygenases (DHBD_{P6-II} and DHBD_{P6-III}), the two isozymes isolated from *Rhodococcus globerulus* P6 and L-DOPA dioxygenase (Lmb1).

The type II extradiol dioxygenases include enzymes consisting of one subunit, e.g. 2,3-dihydroxyphenylpropionate 1,2-dioxygenase (DHPPD_{Eco}) (Spence et al. 1996a), or two different subunits. In some of the type II enzymes, the two different subunits appear to be related, e.g. 2-aminophenol-1,6-dioxygenase (APD_{JS45}) (Davis et al. 1999), while in other cases, they are unrelated, e.g. protocatechuate 4,5-dioxygenase (4,5-PCD) (Sugimoto et al. 1999). The third family of extradiol-type dioxygenases, type III, belongs to the cupin superfamily; dioxygenases belonging to this superfamily include gentisate dioxygenase (GDO), 1-hydroxy-2-naphthoate dioxygenase, hydroxygentisate dioxygenase (HGO) and 3-hydroxy anthranilate 3,4 dioxygenases (HAD) (Dunwell et al. 2001). Some of the important dioxygenases are listed in Table 5.4.

There is no strict correlation between substrate specificity and evolutionary origin in extradiol dioxygenases; thus, there are examples of type I and II HPCDs as well as type I and II C23Os. It is nonetheless noted that the substrate specificity of most ring-cleavage enzymes has not been well investigated and there exists a multiplicity

Table 5.4 Classes of dioxygenases on the basis of their structure

Type	Superfamily	Prototypic members	Divalent ion	Subunit ^a
Intradiol		3,4-PCD _{B-10}		(αβ) ₁₂
		C12O _{ADPI}		α ₂
		HQ12O		α ₂
Extradiol				
Type I	Vicinal oxygen chelate	DHBD _{LB400}		α ₈
		C23O _{mt12}		α ₄
		HPCD _{Bfu}		α _x
		CHQO		α _x
		DHBD _{P6} ^{-II}		α ₆
Type II	Unknown	2,3-dihydroxyphenyl-propionate-1,2-dioxygenase _{Eco}		α ₄
		HPCD _{EcoC}		α _x
		C23O _{JM222} ^{-I}		α _x
		4,5-PCD _{SYK6}		α ₂ β ₂
		APD _{JS45}		α ₂ β ₂
Type III	Cupin	GDO	Fe(II)	α ₄
		1-hydroxy-2-naphthoate-dioxygenase _{KP7}	Fe(II)	α ₆
		HAD	Fe(II)	α ₂

of enzymes: protocatechuate 3,4-dioxygenase (3,4-PCD_{B-10}) from *P. putida* B-10, catechol 1,2-dioxygenase (C12O_{ADPI}) from *Acinetobacter* sp. ADPI, hydroxyquinol 1,2-dioxygenase (HQ12O) from *Nocardioideis simplex* 3E, 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHBD_{LB400}) from *Burkholderia* sp. LB400, catechol 2,3-dioxygenase (C23O_{mt2}) from *P. putida* mt-2, homoprotocatechuate-2,3-dioxygenase (HPCD_{Bfu}) from *B. fuscum*, chlorohydroquinone dioxygenase (CHQO), 2,3-dihydroxybiphenyl 1,2-dioxygenase II (DHBD_{P6-II}) from *R. globerulus* P6⁹, 2,3-dihydroxyphenylpropionate 1,2-dioxygenase from *E. coli*, homoprotocatechuate 2,3-dioxygenase (HPCD_{EcoC}) from *E. coli* C¹²², catechol 2,3-dioxygenase I (C23O_{JMP222-I}) from *Alcaligenes eutrophus* JMP222⁷³, protocatechuate 4,5-dioxygenase (4,5-PCD_{SYK6}) from *Sphingomonas paucimobilis* SYK-6, 2-aminophenol 1,6-dioxygenase (APD_{JS45}) from *Pseudomonas pseudoalcaligenes* JS45, gentisate dioxygenase (GDO) from *Pseudomonas testosteron*, 1-hydroxy-2-naphthoate dioxygenase from *Nocardioideis* sp. KP7, hydroxygentisate dioxygenase (HGO), 3-hydroxyanthranilate 3,4-dioxygenase (HAD). x-oligomeric state is not known.

5.3.3 Structure of Dioxygenases

Crystal structures of Fe(II)-dependent extradiol and extradiol-type dioxygenases are now known for a number of enzymes from *Pseudomonas* and other species

(Li et al. 2006; Ohlendorf et al. 1994; Uragami et al. 2001). These enzymes represent each of the three families of extradiol and extradiol-type dioxygenases and possess the overall structural similarities. The active site of *Brevibacterium fuscum* 2,3- HPCD (a type I structure enzyme) shares some similarities with the type II enzymes protocatechuate 4,5-dioxygenase (LigAB) from *Sphingomonas paucimobilis* and 2,3-dihydroxyphenylpropionate 1,2-dioxygenase (MhpB) from *E. coli*. They share similar active sites His, His, Glu motif for iron (II) cofactor binding and a histidine residue close to the axial coordination site in which dioxygen binds. All have the same iron ligands that constitute the 2-His 1-carboxylate structural motif; several other conserved residues have been identified (Sugimoto et al. 1999; Titus et al. 2000).

5.3.3.1 Type I Extradiol Dioxygenases

All type I extradiol dioxygenases consist of a single type of subunit of size typically 21 and 32.5 kDa for one- and two-domain enzymes, respectively. The majority of type I enzymes identified to date are two-domain, suggesting that the catalytically inactive N-domain confers some sort of advantage to the host. Steady-state kinetic characterisation of one- and two-domain DHBDs in *R. globerulus* P6 suggests that the two domains are catalytically more efficient. It is thus tempting to speculate that the N-domain enables the C-domain to explore a greater range of sequence space, perhaps by conferring additional stability, thereby permitting the evolution of a more efficient active site.

The single-domain enzymes are typically dimeric in structure. While the two-domain enzymes exist in oligomeric state. For example, 2,2',3-trihydroxybiphenyl 1,2-dioxygenase from *Sphingomonas* sp. RW1 is monomeric, C23O_{mt2} is tetrameric and DHBD_{LB400} is octameric (Han et al. 1995). The physiological significance of these different oligomeric states is not understood (Sato et al. 2002).

Sequence alignments and the structure of three enzymes indicate that the tertiary structure of two-domain type I extradiol dioxygenases are very similar even though the overall sequence identities of these enzymes may be less than 15 %. The crystal structure of substrate-free DHBD_{LB400} revealed that each monomer comprises one chain of 297 amino acid residues. Each monomer possesses two domains of very similar structure, the ferrous iron located in the C-terminal domain (C-domain). Each domain is a dimer (2x $\beta\alpha\beta\beta$) made up of four β strands and one α helix forming $\beta\alpha\beta\beta$ modules. Modules 1 and 2 comprise the N-domain and modules 3 and 4 form the C-domain. A large, funnel-shaped space lies entirely within the domain where the active ferrous iron is ligated deep within this space in the C-domain. The C-domain possesses two additional β -strands after the common core structure, and the central funnel is slightly larger than that of the N-domain. The evolutionary adaptation of the two-domain enzymes might have resulted in the loss of a second active site within the N-domain. Catechol 2,3-dioxygenase of *Burkholderia cepacia* and *Pseudomonas putida* mt-2 comprise of two domains that are similar to each other which are separated by a local twofold axis. The N-terminal domain (1–147) contains eight β strands (β 1–8) and two α helices

($\alpha 1$ –2), whereas the C-terminal domain contains nine β strands ($\beta 9$ –17) and four α helices ($\alpha 3$ –6) (Ajao et al. 2012).

The active site for ferrous iron (FeII) is located midway in the 20Å°-long funnel barrel of the C-terminal domain. This funnel is open at both ends; the large opening is 10Å° wide and the smaller opening is 6Å° wide. Thus, the iron is probably only accessible to catecholic substrates from the wide opening, but water or O₂ can access the iron through either end. Iron forms a well-defined square pyramid with His146 as the axial ligand and His210 and Glu260 and two water molecules as equatorial ligands in the basal plane. The active site of catechol 2,3-dioxygenase is capable of interacting and docking with various petroleum hydrocarbons such as benzene, *o*-xylene, toluene, naphthalene, carbazol, pyrene, dibenzothiophene, anthracene, phenanthracene and biphenyl through hydrogen bonds and Van der Waal forces. The crystal structures of DHBD_{kks102} with DHB and DHDB_{LB400} showed that the catechol ring binds in a restricted pocket that is highly complementary in size and shape (Dai et al. 2002; Uragami et al. 2001). One hydroxyl group of the substrate binds in the site trans to His 146, whereas the other binds trans to His 210, displacing the two-ordered water ligands.

Sequence alignments reveal that the one-domain enzymes are similar in structure to the C-domain of type I extradiol dioxygenases. The one-domain enzymes are nevertheless approximately 65 residues larger than the typical C-domain of two-domain enzymes. Though the structure and function of these are unknown, they may be required to stabilise the catalytic domain of these enzymes.

Furthermore, the type I extradiol dioxygenases does not possess an active site in the N-terminal domain. However, the first iron ligand in type I dioxygenases is conserved histidine that is positioned as His146 in DHBD_{LB400}, at the beginning of the first β strand of module 3. This residue is not conserved in HQOs. However, histidine residue is conserved at a similar position in the first β -strand of module 1. It is presumed that the two domains of the HQO type I enzymes comprise of modules 1 and 4 and modules 2 and 3, respectively, and the active site lies within modules 1 and 4.

5.3.3.2 Type II Extradiol Dioxygenases

The type II extradiol dioxygenases are all multimers comprising of one or two different subunit types. For example, 2,3-dihydroxyphenylpropionate 1,2-dioxygenase (DHPPD_{Eco}) (Spence et al. 1996a, b), HPCD_{EcoC} (Roper and Cooper 1990) and C23O_{JMP222-I} (Kabisch and Fortnagel 1990) are homo-oligomers of α subunits. The protocatechuate 4,5-dioxygenases from *Sphingomonas paucimobilis* SKY-6 (4,5-PCD_{syk6}) and 2-aminophenol 1,6-dioxygenase *P. pseudoalcaligenes* JS45 (APD_{JS45}) have heterodimer ($\alpha_2\beta_2$) composition. In the case of 4,5-PCD_{SYK6}, the subunits appear to be unrelated, and the β subunit is similar to the protomers of the homo-oligomeric enzymes. The two subunits of APD share sequence similarity, but only the β subunit contains an active site. Enzyme 4,5-PCD_{syk6} is the only type II enzyme having ferric form as the free-enzyme and forms a binary complex with protocatechuate substrate (Sugimoto et al. 1999). The larger β unit has 302 amino acid residues that form a globular α /

β structure composed of 11 β strands, nine α helices and one 3_{10} helix. They establish α - β contacts and stabilise as $\alpha 2/\beta 2$ dimers which lack α - α and β - β contacts. The active site is located in a cleft in the β subunit on a surface that is extensively covered by the α subunit. The catalytically essential Fe is thus buried and is approximately $15\text{\AA}$ from the surface of the enzyme. In the substrate-free form of the enzyme, the Fe is coordinated in a distorted trigonal pyramidal geometry by His residues $\beta 12$ and $\beta 61$, Glu $\beta 242$ and one water molecule. The protein ligands form the base of the pyramid, and the Fe is displaced from the basal plane towards the water ligand. A weak fifth ligand, Asn $\beta 59$, is located trans to the water at a distance of $2.9\text{\AA}$. Although the protein ligands are identical in character to those observed for the type I enzymes, the three-dimensional arrangement of the ligands is effectively enantiomeric in that one His and Glu ligand exchange locations relative to the positions in DHBD_{LB400} and C23O_{mt2}. Binding of protocatechuate involves both hydroxyl groups and displaces the water ligand. The complex has a distorted trigonal bipyramidal geometry, with his $\beta 61$ and the 3-hydroxyl moiety as axial ligands.

5.3.3.3 Type III Extradiol Dioxygenases and the Cupin Superfamily

In a number of bacterial degradation pathways, intermediates may be converted to gentisate, i.e. a para-diol, rather than to a catecholic compound. Other metabolic pathways involve monohydroxylated aromatic carboxylic acids that directly undergo dioxygenolytic ring-cleavage reactions. Enzymes catalysing these reactions have been referred to as 'type III extradiol dioxygenases', even though most substrates lack the diol character. Examples of these including gentisate 1,2-dioxygenase (GDO), 1-hydroxy-2-naphthoate dioxygenase, hydroxygentisate dioxygenase (HGO) and hydroxyanthranilate dioxygenase (HAD) are part of the cupin superfamily (Dunwell et al. 2001). This superfamily has at least one conserved domain with six antiparallel β -strands that form a β -barrel structure with two distinct motifs. The first motif is composed of the first two β -strands and the second of last two β -strands. The major differences between the various classes of cupins involve variations of the two middle β -strands and the less conserved loop of variable length that separates them. Cupins are functionally diverse and are found in all kingdoms of life. The enzymatic cupins may involve isomerases, epimerases, decarboxylases and many oxygenases. Cupins also include nonenzymatic proteins such as response of plants to pathogens and stresses and are composed of a single domain. Other cupins are composed of two copies of the domain that probably arose from gene duplication, and the examples of this include GO, 1-hydroxy-2-naphthoate dioxygenase, oxalate decarboxylase and seed storage proteins. A special class of cupins is composed of a cupin domain linked to DNA-binding domain. Typical examples of these are AraC/XylS-type transcription factors and some helix-turn-helix transcription factors. In these proteins, the cupin domain binds the effector molecule (Dunwell et al. 2001).

The cupins having a catalytic function often have a metal ion at the active site. The metal ion ligands are present in each of the two β -sheet motifs. These cupins utilise a diverse range of metal ions.

5.3.3.4 The 2-His-1-Carboxylate Metal-Binding Motif

Irrespective of their diversity, all of the extradiol-type dioxygenases contain a 2-His-1-carboxylate metal-binding motif (Hegg and Que 1997; Que and Reynolds 2000). This structural motif is found in a wide variety of unrelated non-haem Fe (II) enzymes, including other microbial catabolic enzymes, indicating its ability to provide a catalytic basis for diverse reactions. The active site of these enzymes contains an Fe(II) ligated by two histidines and one carboxylate all located on one face of the Fe(II) coordination sphere. This motif is thus suitable for ligation of solvent species or molecules at three sites on the other side of the coordination sphere. Apart from the three families of extradiol-type dioxygenases mentioned above, at least three other families of enzymes have been identified to utilise this Fe(II) metal ion site: the ring-hydroxylating dioxygenases, pterin-dependent hydroxylases and α -ketoglutarate-dependent enzymes. This includes some closely related enzymes that do not utilise α -ketoglutarate as a co-substrate. Among the ring-hydroxylating dioxygenases are naphthalene and biphenyl dioxygenases which catalyse the NADH-mediated cis-dihydroxylation of an arene double bond yielding a cis-diol. The Fe^{2+} of these enzymes has two available sites as it is ligated by two oxygens of the carboxylate group. The pterin-dependent hydroxylases are found in mammalian cells, e.g. phenylalanine and tyrosine hydroxylases and use tetrahydrobiopterin as a factor for hydroxylation of these amino acids. A homolog of these is also found in *Pseudomonas aeruginosa* for hydroxylation of phenylalanine to tyrosine (Zhao et al. 1994). The α -glutarate-dependent enzymes are the enzymes involved in synthesis of β -lactam antibiotics. Enzymes like deacetocephalosporin C synthase and clavaminic acid synthase require α -ketoglutarate as a co-substrate to facilitate their reactions.

The mechanism of reaction of these enzymes always involves binding of oxygen atoms to the open sites of the Fe (II) centre. This motif can be seen as the counterpart of the haem cofactor where only one site is available for endogenous ligand compared to three for this motif. The close proximity of the three open sites allows the juxtaposition of the two reactants to promote catalysis. In addition to the enzymes described here, iron superoxide dismutase and lipo-oxygenase also have this structural motif. However, they have an additional histidine ligand, which alters the role of the iron as it shuttles between the Fe(III) and Fe(II) oxidation states during catalysis. The contrast between these two enzymes and the Fe(II) oxygen-activating enzymes shows the flexibility of the 2-His1- carboxylate motif. This flexibility has also been observed in halogenases which possess four open sites for ligation during catalysis. In these enzymes, the carboxylate ligand is replaced by a chloride ion for halogenation of their substrates.

Structure of Intradiol Dioxygenases

The type member of the class is 3,4-PCD_{B-10}, which is large, dodecameric assembly with tetrahedral symmetry (Ohlendorf and Vetting 2001). The crystal structures representing three distinct intradiol dioxygenases have been elucidated and are available in the Protein Data Bank. The protomer comprises two chains of related

structure, α and β , and binds one ferric Fe atom at the active site. The oligomer resembles a porous and hollow, truncated tetrahedron with an edge length of approximately $180\text{\AA}$. Contacts between protomers are mediated by the β subunits which interact across tetrahedral twofold axes to form an inner shell surrounding a central cavity of approximately $50\text{\AA}$ in diameter. The α subunits associate around the threefold axes at each apex and coincidentally lie at the corners of the opposing bases, which have central pore outlined by β -subunits. The crystal structure of a homolog from *Acinetobacter* sp. strain ADP1, 3,4-PCD_{ADP1}, demonstrates the same $(\alpha\beta\text{ Fe(III)})_{12}$ quaternary structure, whereas other 3,4-PCDs utilise the same protomer in a variety of oligomeric states (Vetting et al. 2000).

The α and β subunits of 3,4-PCDs comprise approximately 200 and 230 residues, respectively, and are homologous, and the level of identity between α and β subunits is 30 % for 3,4-PCD_{B-10} and 26 % for 3,4-PCD_{ADP1}. The secondary structure of the subunits is nearly all β and is dominated by a sandwich comprising of eight-stranded sheet folded in half to form two layers.

The active site of each protomer is located at one end of the extensive interface between the α and β subunits near a threefold apex of the oligomer and is accessible from outside the protein. The β chain provides most of the residues in the vicinity of the Fe, although a short segment from near the N-terminus of the α chain (3–4 residues near residue 15) completes the active site. In the substrate-free state of the enzyme, the Fe is bound by one water ligand and four protein side chain ligands supplied by the β subunit, including two tyrosines and two histidines. The ferric iron of the substrate-free intradiol dioxygenase has a trigonal bipyramid geometry, with a tyrosine, a histidine and a solvent moiety coordinated in the equatorial plane and a tyrosine and a histidine in the axial positions. The structures of the binary complexes with substrate and substrate analogs indicate displacement of axial tyrosine accompanies formation of productive complexes. Studies with competitive inhibitors suggest that substrate binding may pass through several stages prior to formation of reactive complex (Orville and Lipscomb 1997).

The crystal structure of C12O_{ADP1} shows another type of structural class of intradiol dioxygenases. This enzyme is a homodimer comprising of a 311 amino acid residue subunit that includes a catalytic domain having a structure similar to the basic core structure of the 3,4-PCDs. The basic core structure is joined to an N-terminal, all-helical dimerisation domain, which includes five helices and approximately 100 residues. The core structure provides four proteins in locations equivalent to those provided by the β subunits in the 3,4-PCDs. An extended segment that links the dimerisation domain to the catalytic domain replaces the short active site segment of the 3,4-PCD α chain. The coordination of the Fe in the substrate-free enzyme is largely the same, and substrate binding displaces the axial tyrosine.

5.4 Mechanism of Catalysis

5.4.1 Extradiol Dioxygenases

Different types of extradiol dioxygenases utilise a similar catalytic mechanism, and the general mechanism is shown in Fig. 5.7. In the first step, the catecholic substrate binds to the ferrous iron in a bidentate manner, displacing the two solvent ligands. Spectroscopic data shows that DHBD_{LB400} binds its preferred substrate, DHB as a monoanion as indicated by X-ray absorption spectroscopic studies on catechol 2,3-dioxygenase (Shu et al. 1995; Vaillancourt et al. 2002). The observed asymmetric binding of DHB to DHBD_{LB400} ($r_{\text{Fe-O}} = 2.0$ and $2.4 \text{ \AA}$) indicates that O_2 is deprotonated. The binding of the catecholic substrate to the iron then activates Fe for O_2 binding. The mechanism involving iron-mediated transfer of an electron from the catechol to the O_2 , yielding a semiquinone-Fe(II) superoxide intermediate, has been substantiated by biochemical studies (Spence et al. 1996b). This results in an iron alkylperoxo-intermediate which undergoes alkenyl migration and O–O bond cleavage to give an unsaturated lactone and an Fe(II)-bound hydroxide ion which hydrolyses the lactone to yield the reaction product.

The crystal structure analysis of various complexes of DHBD_{LB400} and DHBD_{KKS102} suggests roles for the different conserved active sites in the mechanism of catalysis of extradiol dioxygenases. The data strongly suggest that the conserved active site His241 of DHBD_{LB400} assists in the deprotonation of the catechol in the enzyme-catalysed reaction. In the substrate-free enzyme, His241 is

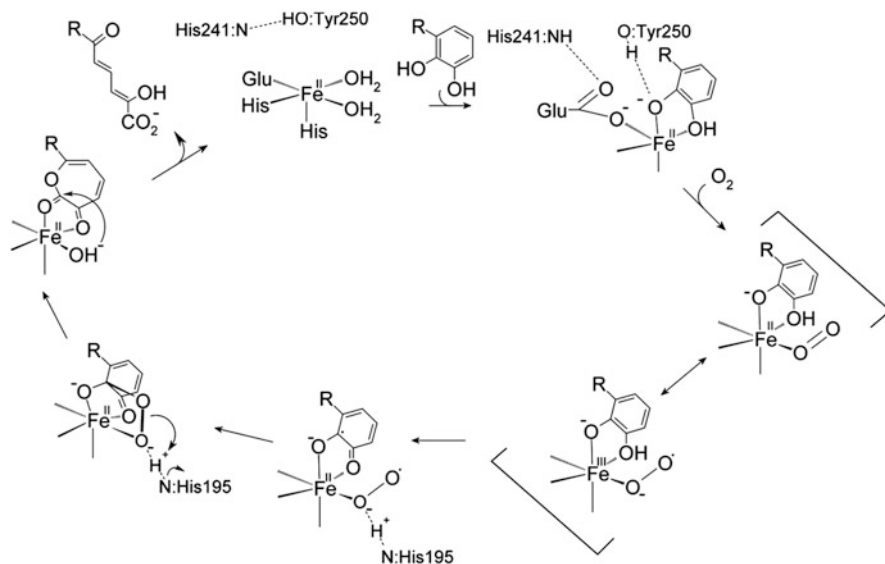
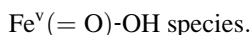


Fig. 5.7 Proposed mechanism of extradiol dioxygenases with the role of conserved active site residues (Bugg and Lin 2001)

presumed to be in the imidazole (neutral) state because of its proximity to the Fe (II) atom and its hydrogen-bonding interactions. The substrate-induced structural changes observed for His241 are consistent with its protonation, the enzyme: DHB complex shows stacking His241 with the catechol ring, and His241 and Glu260 have rotated towards each other forming a new hydrogen bond. This change implies that His241 has acquired a proton during formation of the enzyme-substrate complex. Tyr250 forms a new hydrogen bond with the 2-hydroxyl oxygen of the substrate. Tyr250 probably acts as a proton shuttle between the catecholic substrate and His241.

In the next step of the proposed mechanism, O₂ binds to the ferrous iron which is converted to ferric form as Fe(III)–O₂. This would induce deprotonation of the 3-hydroxyl, forming the dianion chelate, as proposed in intradiol enzymes. The proton could be picked up by the iron-bound superoxide. Recent data implicates a side-on bound iron(III)-hydroperoxy-intermediate during catalysis. Turnover studies of naphthalene dioxygenase reaction has shown that turnover requires reduced enzyme and bound substrate and that one iron(II) and one Rieske(2Fe2S) cluster are oxidised during turnover, resulting in iron(III) at the end of catalytic cycle. Benzoate 1,2-dioxygenase containing a fully oxidised Rieske cluster can utilise hydrogen peroxide to form cis-diol product (Neibergall et al. 2007). These observations are consistent with an iron(III) hydroperoxy-intermediate as active oxidant or which might undergo O–O bond cleavage to form



Kinetic analyses have shown that extradiol dioxygenases are subject to two forms of substrate inhibition, reversible substrate inhibition and a mechanism-based inactivation (or suicide inhibition), as well as oxidative inactivation in the absence of substrate. General mechanism of inactivation in the absence and presence of substrate is quite similar as both involve oxidation of the active site iron. The extradiol-type dioxygenases are susceptible to mechanism-based inactivation by their aromatic substrates such as catechols. Studies on catechol 2,3-dioxygenase (C23O) of *Pseudomonas putida* mt-2 of the TOL pathway have indicated that different catechols inactivate C2,3O to different extents and several mechanisms of inactivation have been proposed. The inactivation of C2,3O by 3-chlorocatechol has been suggested to occur either through reversible chelation of the active site iron or irreversible covalent modification by an acyl chloride species generated by the ring-cleavage reaction. In contrast, the inactivation of C2,3O by alkyl catechols appears to involve the accidental oxidation of the active site Fe(II) to Fe(III) during turnover. The inactivation of C2,3O by 3-methylcatechol may also involve alternate binding modes of the catecholic substrate. The enzyme 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHBD) catalyses the extradiol cleavage of 2,3-dihydroxybiphenyl in the *bph* pathway and is subject to inhibition by 3-chlorocatechol. Mechanism-based inactivation of DHBD_{LB400} in presence of a variety of catechols such as 3-chlorocatechol and DHB involves the dissociation of superoxide.

Reversible substrate inhibition has been reported in a number of enzymes including DHBD_{LB400}, DHBD_{CBI5}, THBD_{RW1}, DHBD_{P6}, DHBD_{BN6} and 2,3-dihydroxyphenyl-propionate dioxygenase. Interestingly, it has only rarely been reported for C₂,₃O_s. The initial rates of cleavage of substituted catechols by DHBD_{BN6} could not be fitted to the substrate inhibition equation. DHBD_{LB400} is clearly subject to both modes of inhibition by both DHB and 3-ethylcatechol. Mechanism-based inactivation of C₂,₃O_{mt2} occurs through reversible chelation of active site iron or irreversible covalent modification of acyl chloride species generated by ring-cleavage reaction.

Extradiol oxygenases are also sensitive to oxidative inactivation in the absence of substrate. This involves binding of the oxygen to the active site iron and loss of superoxide. This mechanism might have evolved as a protective mechanism against oxidative inactivation. Enzyme C₂,₃O_{mt2} has a lower K_{mO_2} and is less susceptible to oxygen-dependent inactivation. The oxidative inactivation of extradiol dioxygenases in the absence of substrate complicates their purification and characterisation.

5.4.2 Intradiol Dioxygenases

The mechanism of catalysis by intradiol dioxygenases has been developed on the basis of biochemical, spectroscopic and structural studies of 3,4-PCDs and C₁₂O_s (Bugg and Lin 2001). Like extradiol dioxygenases, the intradiol enzymes utilise an ordered mechanism in which catechol binding precedes O₂ reactivity. However, whereas extradiol enzymes activate the O₂ for nucleophilic attack on the catechols, the intradiol enzymes function to activate the catechols for electrophilic attack by O₂. In intradiol dioxygenases, catechol binding is a multistep process that ultimately results in displacement of an axial tyrosine and an equatorial hydroxide ion to yield a bidentate-bound catecholate. It is generally accepted that the displaced tyrosyl and hydroxide ligands accept the two hydroxyl protons of the substrate such that the latter binds as a dianion. In the next step, O₂ attacks the bound catecholate directly before coordinating to the iron moiety and yielding an iron-alkylperoxo-intermediate. Recent evidence indicates that this intermediate is structurally similar to that of the extradiol reaction; however, in the case of intradiol enzymes, the Criegee rearrangement and O–O bond cleavage involve acyl migration to yield the cyclic anhydride and an iron-bound oxide or hydroxide. The latter functions as a nucleophile to hydrolyse the anhydride and yield the ring-opened product. The crystallographic studies indicate that the substrate is asymmetrically bound: the long Fe–O bond is in transposition to tyrosine ligand and the short Fe–O bond is trans to histidine ligand. The asymmetry is considered to reflect ketonisation of the bond trans to tyrosine. 4-Nitrocatechol is an inhibitor of 3,4-PCD, and 3,4-dihydroxyphenylacetate is a very poor substrate for the enzyme. Thus, these analogs do not bind in the same manner as PCA, the preferred substrate of the enzyme. However, structural data indicate that 3,4-PCD binds 3, 4-dihydroxyphenylacetate and PCA in a similar manner. Analysis of the dianionic

binding of the substrate to substrate activation in the catalytic mechanism of intradiol enzymes perhaps could provide more information of protonation state of the bound substrate (Vetting and Ohlendorf 2000; Winfield et al. 2000).

5.4.3 Comparative Mechanism and Specificity of Dioxygenases

The orientation of the iron-alkylperoxo-moiety relative to the organic substrate is of key importance in cleavage of the common intermediate by intradiol and extradiol dioxygenases. In particular, the extradiol dioxygenases are proposed to form a pseudo-axial iron-alkylperoxo species that would favour alkenyl migration and the intradiol dioxygenases are proposed to form a pseudo-equatorial iron-alkylperoxo species that would favour acyl migration. The hypothesis suggesting that extradiol cleavage involves alkenyl migration whereas intradiol cleavage involves acyl migration is supported by the range of compounds that are known substrates for these enzymes. Assuming that ring cleavage by both types of these enzymes proceeds via a similar iron alkylperoxos-intermediate, acyl migration can occur only in the known substrates of intradiol dioxygenases, e.g. catechols and substituted derivatives thereof. In contrast, the cleavage of compounds such as gentisate, which do not have vicinal hydroxyl groups, can only proceed via alkenyl migration, consistent with the extradiol dioxygenase mechanism.

Important difference lies in the initial stages, i.e. the protonation state of the bidentate-bound catechol in the enzyme/substrate complex. In the extradiol dioxygenases, a monoanionic Fe(II)-bound catecholate activates the ferrous centre for O₂-binding, and, in intradiol dioxygenases, a dianionic Fe(III)-bound catecholate promotes direct electrophilic attack of the substrate by O₂. Although the different protonation states of the substrate in the two enzymes have been considered important, the evidence for dianionic binding in the intradiol dioxygenase is not conclusive. As already pointed out, the structural data suggest that in both the intradiol and extradiol dioxygenases, the substrate is asymmetrically bound, one of the Fe–O bond is shorter than the other. This has been interpreted as ketonised dianion in the intradiol dioxygenases. Considering that extradiol cleavage is more difficult to achieve than the intradiol cleavage, it has been suggested that intradiol cleavage is a ‘default pathway’ (Bugg and Ramaswamy 2008). Since both iron(II) and iron(III) can catalyse formation of extradiol and intradiol products, the oxidation state of iron is not crucial in the choice of the pathway. For extradiol cleavage, the hydroperoxide intermediate is bound in tridentate manner to the iron(II) centre for stereoelectronic migration of alkenyl migration. While for intradiol cleavage, hydroperoxide intermediate is bound in bidentate fashion to the iron(III) centre so that the C–C is not suitably arranged for acyl migration and the absence of nearby acid-base residues raises the possibility of O–O homolysis during the rearrangement (Borowski and Siegbahn 2006). The extradiol dioxygenases have implications for the engineering of pathways for degradation of environmental pollutants.

5.5 Applications of Oxygenases

Many enzymes like oxygenases, reductases, dehydrogenases, dehalogenases and hydroxylases are involved in bacterial biodegradation of organic compounds (Karigar and Rao 2011). Among these, oxygenases play an important role in bacterial degradation of hydrocarbons as most often they are the initiators to catalyse the degradation reactions. They form a highly diverse group of enzymes which differ in structure, mechanism and the requirement of the coenzyme. Oxygenases are excellent biocatalysts involved in various synthetic and transformation reactions. They incorporate oxygen atom(s) into substrate. They comprise a versatile superfamily of enzymes that catalyses oxidative reactions of substrate ranging from alkanes to steroids, fatty acid, etc. Because of their functional roles, they offer potential in bioremediation technology. They are actively involved in dehalogenation, desulphurisation, denitrification and hydroxylation of various aromatic compounds (Arora et al. 2010) and serve in the remediation of pollutants from the environment due to their high regio-, stereo- and enantioselectivity on a wide range of substrates. A number of toxic substrates are utilised by various oxygenases. This family of enzymes actively contributes in the degradation of environmental pollutants like substituted methanes, alkanes, cycloalkanes, alkenes, haloalkenes ethers, heterocyclic compounds, etc. (Karigar and Rao 2011). Several microorganisms capable of degrading highly toxic compounds such as polychlorinated dibenzofurans or dibenzo-*p*-dioxins have been identified. These compounds occur as unwanted products during incineration of municipal waste and chemical pesticide and herbicide industry. The enzyme involved in their degradation is referred as angular dioxygenase as the enzyme acts on the angular position adjacent to the oxygen atom of dibenzofuran and dibenzo-*p*-dioxide (Habe et al. 2001). Besides environmental applications, oxygenases hold great potential in areas such as textiles, food, biosensors, organic synthesis (chiral and asymmetric synthesis, polymers, pharmaceuticals) and biofuels (Xu 2005). Various oxidised aromatic compounds can be efficiently synthesised using oxygenases (Nolan and O'Connor 2008). Furthermore, protein engineering of oxygenases and random mutagenesis offers the potential of improving the catalytic activities (Brouk et al. 2010; Dror and Fishman 2012).

5.6 Conclusion

No doubt oxygenases found in *Pseudomonas* are multifunctional enzymes and hold a lot of potential up to industrial scale. Biotechnological tools have helped to further exaggerate its beauty and potential. A lot of studies have been done for these Gram-negative bacteria, but a lot has to be explored. As far as environmental pollution is concerned, *Pseudomonas* is able to degrade almost all types of pollutants with the aid of its precious wealth of enzymes. These enzymes are also able to transform various molecules into industrially important compounds which are chemically synthesised and are very costly, as well as the process and reagents required for

the synthesis create environmental hazards. Several transformations are carried out by this genera, and the use of recombinant techniques has increased its efficiency several folds whether related to biodegradation or biotransformation, where oxygenases play a very important role. Catechol and chlorinated catechols are considered to be carcinogenic and teratogenic. These, therefore, need to be removed from the environment. Catechol dioxygenase and chlorocatechol dioxygenase can play an important role in detoxification/degradation of such pollutants. But still, there is a need to further look into the loopholes which have to be cleared to fully explore the enzyme's specificity, selectivity and versatility. Thermostability of the enzymes under industrial conditions is an important issue for their application. Introduction of the disulphide in catechol 2,3 dioxygenase of *Pseudomonas* sp. CGMCC2953 resulted in improved thermostability of the enzyme. There appears to be lot of opportunity to modify and exploit the potential of oxygenases as 'biocatalyst' for human welfare.

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Quorum Sensing in *Pseudomonas aeruginosa*: Mechanism and Regulation of Virulence

6

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Contents

6.1	Introduction	232
6.1.1	Bacterium	232
6.1.2	Infections and Clinical Management	233
6.2	Quorum Sensing: The Key Regulator	234
6.2.1	Las System	235
6.2.2	Rhl System	235
6.2.3	PQS System	237
6.2.4	IQS System	237
6.3	Network of QS Systems	238
6.4	Global Regulation of Quorum Sensing	239
6.4.1	Stationary Phase Sigma Factors (RpoS/RpoN)	239
6.4.2	RsaL and MvaT	241
6.4.3	QscR: Orphan Regulator	241
6.4.4	VqsR	241
6.4.5	RsmA	242
6.4.6	DksA	242
6.4.7	Vfr	242
6.4.8	Polyphosphate Kinase (PPK)	242
6.4.9	Two Component Regulatory Systems	243
6.5	Quorum-Sensing-Regulated Virulence Factors in <i>P. aeruginosa</i>	244
6.5.1	Proteases	244
6.5.2	Pyocyanin	245
6.5.3	Rhamnolipids	246
6.5.4	Bacterial Motilities	247
6.5.5	Biofilm	248
6.6	Quorum Quenching	250
	References	251

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Abstract

P. aeruginosa is the causative agent of nosocomial infections, especially in immunocompromised and burn patients and causes chronic infections in cystic fibrosis patients. The intrinsic resistance of *P. aeruginosa* against different antibiotic groups led to the emergence of multiple drug resistance thereby making its eradication difficult. An array of cell associated and extracellular virulence factors have made this organism capable to inhabit any type of tissue. Quorum sensing is the global regulatory network in *P. aeruginosa* capable of regulating the expression of virulence and hence its pathogenicity. There are a total of four QS systems in *P. aeruginosa*, viz., LasIR, RhlIR, PQS and IQS, interconnected to each other through various pathways, hence regulating the expression of various target virulence genes. The interference in QS system attenuates *P. aeruginosa*, hence making its eradication more easy. This strategy has been emerged as an alternative method of targeting *P. aeruginosa* infection and the basis of future medicines.

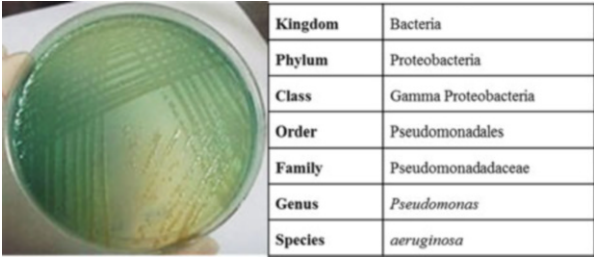
6.1 Introduction

P. aeruginosa is a member of normal microflora of human body but gets virulent if host defence is compromised. In fact, *P. aeruginosa* is the epitome of an opportunistic pathogen of humans. The bacterium almost never infects uncompromised tissues, yet there is hardly any tissue that it cannot infect. According to a detailed hospital survey conducted by the *P. aeruginosa* was the sixth most prevalent causative agent in healthcare-associated infections and was responsible for 14 % of pneumonia cases, 7 % of surgical site and urinary tract infections, 2 % of blood stream infection and 1 % of intestinal infections. In the past decade, *P. aeruginosa* prevalence has increased with the development of resistance to 23 antibiotics making this organism a definite threat for susceptible patients (Aloush et al. 2006; Edwards et al. 2007; Joseph et al. 2013). The remarkable ability of *P. aeruginosa* to colonize human host system owes to the array of virulence factors it produces and their alternative mode of growth as biofilm.

6.1.1 Bacterium

P. aeruginosa is a member of the class Gamma Proteobacteria (Fig. 6.1). It is a Gram-negative, aerobic rod measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm belonging to the bacterial family Pseudomonadaceae. It is a free-living, motile (by means of a single polar flagellum), oxidase-positive, non-sporulating and non-fermentative species (Bergey 2001). In laboratory conditions, it forms smooth and mucoid colonies on Luria-Bertani medium (Fig. 6.1). It occurs regularly on the surfaces of plants and is studied for its ability to colonize plant niches. Other strains of *Pseudomonas* like *P. fluorescens*, *P. putida*, *P. aureofaciens* and *P. chlororaphis*

Fig. 6.1 *Pseudomonas aeruginosa* visible as greenish blue colonies when grown on Luria Bertani medium



reside in rhizosphere where they act as plant beneficial bacteria and clear off other notorious disease-causing bacteria. However, *P. aeruginosa* has emerged as an important opportunistic pathogen of clinical relevance.

6.1.2 Infections and Clinical Management

P. aeruginosa causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immunosuppressed (Krcmery et al. 2006; Regules et al. 2008). Neonates are also susceptible to *P. aeruginosa* infection due to underdeveloped immune system (Foca 2002). The major implication of *P. aeruginosa* is severity of pulmonary infections in diffuse panbronchiolitis and chronic obstructive pulmonary patients. The ultimate sufferers are cystic fibrosis patients where 85–90 % of them succumb to the consequences of *P. aeruginosa* infections (Gibson et al. 2003).

Treatment of *P. aeruginosa* infections depends on their severity and the extent to which pathogenesis has been established. β -lactam is the most common group of antibiotics that have been used for treatment of *P. aeruginosa* infections. However, *P. aeruginosa* has developed an intrinsic ability to degrade β -lactam antibiotics, genes of which are carried either on plasmid (metallo- β -lactamases) or chromosome (*ampC*) or both. The genes for antibacterial resistance are carried through horizontal gene transfer. Also there is an increased expression of efflux pump (MexAB OprM, MexCD OprJ, MexEF OprN, MexXY OprM), loss of porin OprD and impermeability of membrane that makes *P. aeruginosa* resistant to almost every class of drugs leading to emergence of strains with multiple drug resistance.

In the current medical scenario, to combat infections by MDR strains, patient is administered with anti-pseudomonal cocktails that include combinations of a β -lactam such as ticarcillin, aztreonam or ceftazidime plus a β -lactamase inhibitor such as sulbactam and an aminoglycoside antibiotic such as tobramycin or amikacin (Flanders et al. 2006; Ostendorf et al. 2006). Alternative combinations include pairing ciprofloxacin with an aminoglycoside or with the broad-spectrum antibiotic fosfomycin, which prevents proper cell wall formation by inhibiting the production of *N*-acetylmuramic acid (Flanders et al. 2006). Polyamines like

cadaverine, putrescine, spermidine and spermine enhance the activity of nalidixic acid, trimethoprim and 14 different β -lactam antibiotics in vitro and are regarded as effective therapy against *P. aeruginosa* infection.

The use of more modified antibiotics will keep on increasing the chances of developing MDR strains. Recently, Clinafloxacin was synthesized which showed slightly more activity than ciprofloxacin, but its development has been suspended due to its toxicity. For the long term, multidrug efflux inhibitors are promising for use with fluoroquinolones or β -lactams and metallo- β -lactamase inhibitors, but unless new drugs are not developed, it is hard to escape the conclusion that multidrug-resistant *Pseudomonas* strains will be an unmanageable reality.

Meanwhile, there was development of new concepts of creating anti-pathogenic drug that could control the pathogenicity of the microbe without killing the organism. In this regard, disruption of bacterial cell communication system known as quorum sensing (QS) has been found to be an attractive target that will attenuate *P. aeruginosa* and will lead to effectively eradication by host immune system.

Thereafter, a whole lot of research was carried out to understand the QS system in *P. aeruginosa*, its regulation and virulence factors under its control.

6.2 Quorum Sensing: The Key Regulator

Fuqua et al. (1994) was discovered an intercellular communication system in bacterial cells. They produce small diffusible (or efflux pumps mediated) autoinducer molecules (encoded by a synthase gene), and when these signal molecules reach a particular threshold concentration, they bind to their cognate transcriptional regulator, and the targeted genes are either repressed or activated. Thus, genes are expressed in a coordinated manner in accordance with the population density which is termed as quorum sensing (QS) (Fig. 6.2). More precisely, bacteria will express genes only when quorum is achieved. Bacteria need quorum sensing to show population-based physiology.

In the case of pathogenic bacteria, single cell can hardly overcome the dynamic antimicrobial host immune system, while when these pathogens express their virulence after attaining a threshold population, they become more virulent and capable to invade host immune system. QS signaling system in *P. aeruginosa* is considered to be a global regulator affecting the expression of 353–616 genes (10 % of total genes) including those essential for pathogenesis (Whiteley et al. 1999). There are a total of three QS systems reported in *P. aeruginosa* where two are *N*-acylhomoserine lactone mediated while the third is 2-heptyl-3-hydroxy-4-quinolone as the QS signal. Recently, a fourth QS system has been identified termed as IQS system (Table 6.1).

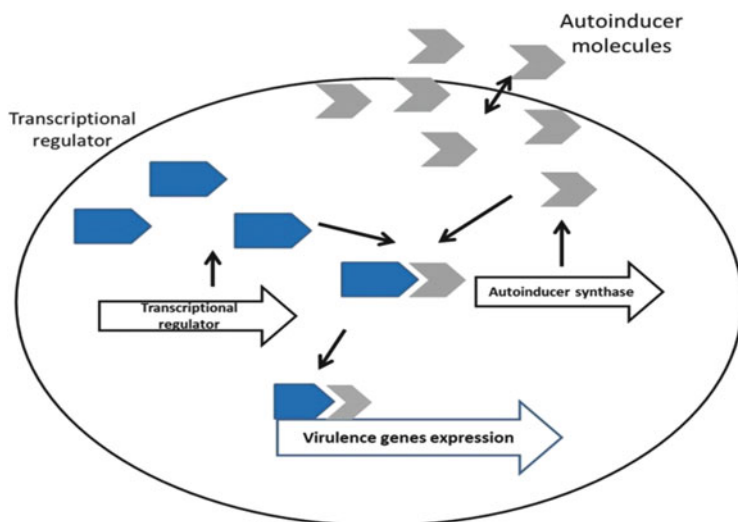


Fig. 6.2 Schematic representation of quorum-sensing mechanism

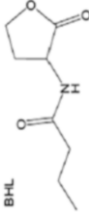
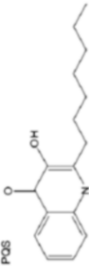
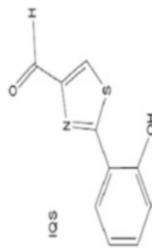
6.2.1 Las System

The Las system utilizes *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (3-oxo- C_{12} -HSL) (Fig. 6.3) as its signal molecule synthesized by autoinducer synthase LasI. It is transported out of the cell; enters back to bacterial cell when threshold concentration is achieved; interacts with its transcriptional regulator, LasR; and activates promoters of the target genes. Las system gets activated during early log phase, and *lasR* gene is expressed throughout the growth phase. LasR interacts with 3-oxo- C_{12} -HSL and multimerizes and positively regulates *lasI* expression, creating a positive induction loop (Seed et al. 1995). LasR/3-oxo- C_{12} -HSL also induces expression of other PQS and Rhl systems during early and late stationary phase. LasR forms a multimer and binds *las* boxes present in the promoter of target genes and essentially requires 3-oxo- C_{12} -HSL for activation (Kiratisin et al. 2002). Las system exclusively regulates the expression of elastase (*lasB*), alkaline protease (*lasA*) and exotoxin A (Smith and Iglewski 2003).

6.2.2 Rhl System

In RhlIR QS system, RhlI directs the synthesis of *N*-(butanoyl)-L-homoserine lactone (C_4 -HSL) (Fig. 6.3), which is able to freely diffuse across the cell membrane and interact with the cognate regulator RhlR. Unlike *las* system, RhlR dimerization is independent of C_4 -HSL, and it binds to the *rhl* boxes in the promoter of virulence genes (Medina et al. 2003; Ventre et al. 2003). *Rhl* system activation occurs in the late stationary phase and maintains the pathogenicity of *P. aeruginosa*

Table 6.1 QS systems in *P. aeruginosa* PAO1

QS system	Autoinducer signal		Transcriptional regulator	Virulence factors
	Molecule	Structure		
LasIR	N-(3 oxo dodecanoyl)-homoserine lactone		LasR	Elastase, protease, biofilm
RhIIR	N-butyryl homoserine lactone		RhIR	Rhamnolipids, pyocyanin, swarming, biofilm
PQS	2-heptyl-3-hydroxy-4-quinolone		MvfR	Pyocyanin, pyoverdine, rhamnolipids
IQS	2-(2-hydroxy phenyl)-thiazole-4-carbaldehyde		Not defined	Not defined

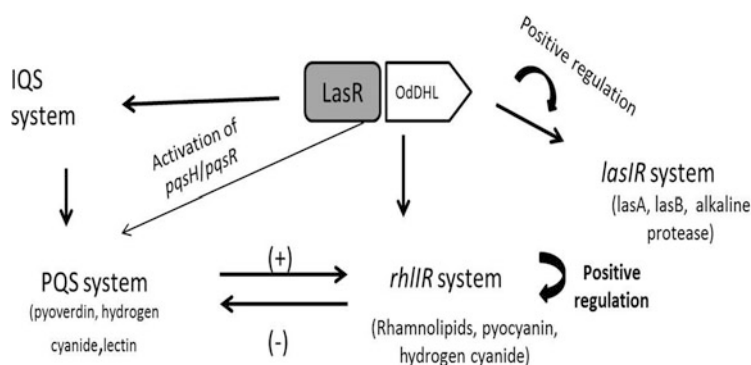


Fig. 6.3 QS systems in *P. aeruginosa* and their network

during stress condition. RhlIR exclusively regulates the production of rhamnolipids, pyocyanin, lectins and superoxide dismutase in *P. aeruginosa*.

6.2.3 PQS System

P. aeruginosa also expresses another QS system termed as *Pseudomonas* Quinolone Signal system (PQS) (Fig. 6.3) (Pesci et al. 1999). PQS (2-heptyl-3-hydroxy-4-quinolone) is the autoinducer molecule that binds to its transcriptional regulator PqsR and regulates the synthesis of other genes, i.e. *phnAB* (phenazines) and *pqsABCDE* (PQS signal molecules) operons.

PQS cannot diffuse out of the cell easily. Hence, HHQ (4-hydroxy-2-heptyl-quinoline), the immediate precursor of PQS, is thought to be released by a cell in a bacterial population which is taken up by another cell and converted into PQS through the gene product of *pqsH* (Deziel et al. 2004). The activation of *pqsH* transcription depends on LasR/3-oxo-C12 HSL dimer which consecutively directs the synthesis of PQS in the cell and thus the activation of PQS system. PQS is expressed in the late stationary phase and does not involve in sensing signal density. Perhaps it induces RhlIR system transcriptionally, for gene expression in stationary phase (McKnight et al. 2000). PQS system regulates expression of many virulence factors through activating Rhl system like pyoverdine, rhamnolipids and elastase. These virulence factors are needed by the pathogenic bacteria during late stationary phase period because they help in increasing the nutrient availability by tissue destruction in vivo (Pesci et al. 1999; Calfee et al. 2001; Diggle et al. 2003; Deziel et al. 2004, 2005).

6.2.4 IQS System

IQS system (Integrated Quorum-Sensing Signal) is the fourth intercellular communication signal system identified in *P. aeruginosa* which is capable of integrating

environmental stress signals with the quorum-sensing network (Lee et al. 2013). The signal molecule was structurally identified as 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (Fig. 6.3) and the genes which are involved in the synthesis of signal molecule are non-ribosomal peptide synthase gene cluster *ambBCDE*. Interference in the synthesis of signal molecule leads to decrease in the production of PQS and BHL (C4-HSL) signals which consecutively leads to reduction in pyocyanin, rhamnolipids and elastase. Importantly, under phosphate depletion stress conditions, IQS partially took the functions of the central Las system (Lee et al. 2013). IQS mutants were unable to cause *P. aeruginosa* infection in the four different animal models (mouse, zebrafish, fruit fly and nematode), highlighting the important role of this new QS system in modulation of bacterial pathogenesis.

6.3 Network of QS Systems

The four QS systems are well organized and arranged in multi-layered hierarchy. At the top of this global QS network is the Las system, and the basic theory of QS activation goes like this: some basal expression of *lasI* leads to weak production of 3-oxo-C₁₂ HSL (OdDHL) which binds to some basal lasR regulators. LasR-3-oxo-C₁₂ HSL (OdDHL) complex multimerizes and activates the transcription of rhIR, rhII and lasI, hence creating a positive feedback loop. This complex also activates the transcription of other virulence genes (proteases, toxins) that are part of its regulon (Kiratisin et al. 2002; Pesci et al. 1997). Meanwhile, rhI system gets activated, and complex of RhIR-C4 HSL binds and activates the virulence genes of its own regulon and rhII, creating the second feedback loop.

LasR-3-oxo-C₁₂ HSL also activates PqsR, the transcription regulator of HHQ/PQS biosynthetic operon *pqsABCD*, and *pqsH* gene which encodes the enzyme required to convert HHQ to PQS (Deziel et al. 2004; Gallagher et al. 2002; Xiao et al. 2006).

As soon as pqs signal production starts, it upregulates the transcription rate of rhII, thereby increasing the expression of virulence factors mediated by rhIR system (McKnight et al. 2000; Pesci et al. 1999). On the contrary, *pqsR* and *pqsABCDE* expression is inhibited by the complex RhIR/BHL (Cao et al. 2001), thus indicating the ratio of OdDHL and BHL plays a decisive role in the dominance of the pqs signaling system (Cao et al. 2001). Since LasR-OdDHL turned out to be main leader that controls the onset and activation of both the pqs and rhI QS circuits, these systems therefore represent a step-wise activation cascade that will be triggered only when certain “quorum” is reached by *P. aeruginosa* culture.

However, in a later study, it was found that RhIR may overtake the function of LasR when the later is absent and regulates the transcription of both *las*- and *rhI*-mediated virulence gene expression along with the activation of Pqs (Dekimpe and Deziel 2009).

The recently identified IQS was found to be controlled by LasIR when bacteria were allowed to grow under rich medium conditions (phosphate-rich medium).

Disruption of either *lasR* or *lasI* completely disrupts the expression of *ambBCDE* and the production of IQS (Lee et al. 2013). Also if bacteria are allowed to grow in phosphate-depleted medium, *las* system fails but *iqs* upregulates the expression of *rhl*- and *pqs*-mediated virulence.

QS system hierarchy shows rapid changes depending on the various environmental cues, thus suggesting that the main role of bacterial QS systems is to modulate bacteria so that they could persist and express their full pathogenicity irrespective of any adverse condition. Schematic representation of the QS regulatory circuit has been given in Fig. 6.3 for clarity.

6.4 Global Regulation of Quorum Sensing

Quorum sensing in *P. aeruginosa* itself is under regulation of global factors which act as checkpoints as to when, how and which QS system has to get activated or repressed. These global regulators help *P. aeruginosa* to inhabit a wide range of biological niches, and this is supported by the presence of large proportion of regulatory cascades in the genome. Quorum-sensing system is well integrated to other global circuits so that not only the cell density but the environment conditions should also be well suited to bacteria to show their virulence.

QS regulation occurs via transcriptional and/or post-transcriptional regulation of AHL synthase and transcription regulator. The stability and activity of transcriptional regulators also act as the regulatory targets along with the autoinducer molecules. Some of the major regulators include the cAMP receptor regulatory protein Vfr, the stationary-phase sigma factor RpoS, the alternative sigma factor RpoN, the stringent response protein RelA, two LuxR homologues (QscR and VqsR), the posttranscriptional regulators RsmA and DksA, the transcriptional regulatory proteins RsaL and MvaT and the global two component regulatory system GacA–GacS (Table 6.2).

6.4.1 Stationary Phase Sigma Factors (RpoS/RpoN)

RpoS (σ^s or σ^{38}) is a central regulator which responds to different stresses and leads to cessation of growth, thus bringing the cells to stationary phase (Hengge-Aronis 2002). RpoS regulates the synthesis of alginate, pyocyanin and exotoxin A which are also under QS regulation. This overlap between the two regulatory cascades was proved by transcriptomics analysis where approximately 30–40 % of genes were found to be controlled by both QS and RpoS (Schuster et al. 2004). However, the exact link between the two has not been determined yet.

The alternative sigma factor RpoN (σ^{54}) regulates the virulence of *P. aeruginosa* mainly pili and fimbriae formation required for motility. The mode of action of RpoN for the regulation of QS has not been deciphered; however, it has been suggested that RpoN could regulate other regulatory components of AHL QS cascade.

Table 6.2 Global regulators of quorum sensing in *P. aeruginosa*

Regulators	Targets	Remarks	References
RpoS	Negative transcriptional regulator of <i>rhlI</i>	Considerable overlap between RpoS and QS regulons	Latifi et al. (1996), Whiteley et al. (2000), Schuster et al. (2004)
RpoN	Negative transcriptional regulator of <i>lasIR</i> and <i>rhlIR</i>	Minimal medium RpoN positively regulates <i>RhlI</i> expression	Heurlier et al. (2003), Thompson et al. (2003)
RsaL	Negative transcriptional regulator of <i>lasI</i>	In competition with <i>LasR</i> /3-oxo-C12-AHL for <i>lasI</i> transcription, Avoids early activation of QS	De kievit et al. (1999), Bertani and Venturi (2004)
MvaT	Negative transcriptional regulator	Avoids early activation of QS	Diggle et al. (2002)
QscR	Negative regulator of <i>LasR</i> protein	Avoids early activation of QS, <i>LuxR</i> family member	Chugani et al. (2001), Ledgham et al. (2003b)
RsmA	Negative transcriptional regulator of <i>lasI</i>	Avoids early activation of QS	Pessi et al. (2001)
DksA	Negative transcriptional regulator of <i>rhlI</i>	Avoids early activation of QS	Branny et al. (2001), Jude et al. (2003)
Vfr	Positive transcriptional regulator of <i>lasR</i> and <i>rhlR</i>	Binds to <i>lasR</i> promoter, not known if it binds to <i>rhlR</i>	Albus et al. (1997)
VqsR	Positive transcriptional regulator of <i>lasI</i>	Considerable overlap between <i>VqsR</i> and QS regulons, <i>LuxR</i> family member	Juhas et al. (2004)
GacA–GacS	Positive transcriptional regulator of <i>lasR</i> and <i>rhlR</i>	Two component signal transduction system, environmental stress unknown	Reimmann et al. (1997), Parkins et al. (2001)
PprB	Positive transcriptional regulator of <i>rhlI</i> and <i>rhlR</i>	Response regulator of Two component signal transduction system, overlap of <i>PprB</i> and QS regulons, environmental stress unknown	Dong et al. (2005)
PQS	Positive transcriptional regulator of <i>rhlI</i>	PQS is a signal molecule; its production is regulated by <i>Las</i> system	Pesci et al. (1999), McKnight et al. (2000), Gallagher et al. (2002), Diggle et al. (2003)
AlgR2	Negative transcriptional regulator of <i>lasIR</i> and <i>rhlIR</i>	Also major regulator of alginate production; homologues of <i>E. coli</i> <i>Rsd</i> protein which is an anti- σ 70 factor	Ledgham et al. (2003a), Westblade et al. (2004)
PPK	QS, biofilm, motility	Essential for stress survival	Fraley et al. (2007)
Qsro	Inhibits <i>las</i> , <i>rhl</i> , <i>pqs</i>	Genetic locus ORF2226	Köhler et al. (2014)

6.4.2 RsaL and MvaT

In *P. aeruginosa*, *lasR* and *lasI* are situated on the same DNA strand separated by 367 bp intergenic sequence and have their own individual promoters. Within this intergenic sequence lies the *rsaL*, on the strand opposite to *lasR*, which negatively regulates the transcription of *lasI* by binding to the promoter (de Kievit et al. 1999). Interestingly, *lasR* induces the expression of RsaL. RsaL binds to the bidirectional *rsaL-lasI* promoter and inhibits the expression of both genes (i.e. *rsaL* and *lasI*) generating a negative feedback loop that counteracts the positive signal feedback loop of LasR-3-oxo-C₁₂ HSL/*lasI*, thereby balancing the levels of 3-oxo-C₁₂ HSL (Rampioni et al. 2007). Both LasR/OdDHL and RsaL bind to the same promoter site of *lasI*, but they do not compete with each other. However, the repression by RsaL is stronger than the activation by LasR (Rampioni et al. 2007). RsaL also inhibits the expression of some QS target genes such as biosynthetic genes of pyocyanin and cyanide (Rampioni et al. 2007).

Mva T has been implicated to play an important role in transcriptional repression of QS at low cell densities. MvaT mutant showed early expression of QS showing increased expression of AHLs, pyocyanin and elastase production (Diggle et al. 2002) suggesting its role as repressor. Through homology study, MvaT showed structure similarity with H-NS-like protein which controls the expression of genes involved in metabolism and environmental adaptation (Hommals et al. 2001). MvaT also positively regulates *cupA* protein (chaperone usher pathway) required for assembly of fimbrial structure and biofilm formation (Vallet et al. 2004).

6.4.3 QscR: Orphan Regulator

QscR shows a remarkable similarity with the QS transcriptional regulator but lacks any cognate autoinducer synthase gene. QscR mutant showed early expression of QS-mediated genes at very low cell density compared to wild type (Chugani et al. 2001; Ledgham et al. 2003b). Hence, QscR is involved in the repression of *lasI*, which retards the production of 3-oxo-C₁₂ HSL, and since this AHL and *lasR* are activators of *rhl* system, repression of *lasIR* also retards *rhl* system.

6.4.4 VqsR

VqsR, a homologue of transcriptional regulator, is an important positive regulator of QS whose direct targets remain unknown. *P. aeruginosa vqsR* mutant displayed reduced production of quorum signals (ASLs) and virulence factors and reduced pathogenicity in nematode model relative to the wild type (Juhas et al. 2004). Microarray analysis revealed that VqsR controls the expression of approximately 200 genes and 50 % of them showed overlapping of the regulon (Juhas et al. 2005). VqsR gene contains the putative *las* box in its promoter region, which could be the

probable explanation of the overlapping of QS regulon (Juhas et al. 2005). VqsR indirectly controls *las* or *rhl* systems via regulating the transcription of *lasI*, but directly regulates the LasR-RhlR homologue QscR (Liang et al. 2012).

6.4.5 RsmA

RsmA is a 7 kDa RNA-binding global regulator protein Repressor of Secondary Metabolism and is highly conserved in eubacteria. It regulates the production of secondary metabolites post-transcriptionally by acting as a translational repressor which probably binds to the ribosome binding site leading to the unstable mRNA. In *P. aeruginosa*, the repression by Rsm A is regulated by small regulatory RNAs whose expression is known to be under global two component system GacA–GacS (Heeb et al. 2002). The Rsm A mutant showed high levels of AHLs indicating its role as negative regulator. However, exact mechanism of Rsm A repression is not known.

6.4.6 DksA

DksA is a negative regulator of QS and acts on the *rhl* system by downregulating the transcription of *rhlI*. It has been shown that overexpression of *dksA* in *P. aeruginosa* resulted in inhibition of *rhlI* transcription and hence *rhl*-related virulence expression (Branny et al. 2001). The exact mechanism through which this regulation occurs is still to be elucidated.

6.4.7 Vfr

Vfr showed 91 % structural similarity with the cAMP receptor protein (CRP) of *E. coli*. It was also found to regulate the production of virulence factors (elastase and proteases) in *P. aeruginosa* by interacting with RNA polymerase (West et al. 1994). Vfr directly regulates the transcription of *lasR* which then triggers the expression of various virulence factors and *rhl* system (Albus et al. 1997). Vfr binds to the transcriptional start site of both *lasR* and *rhlR*, different from their normal *las* and *rhl* boxes. However, it is still unclear whether Vfr directly activates *rhlR* promoter through a Vfr binding site or indirectly via another regulators.

6.4.8 Polyphosphate Kinase (PPK)

In the recent study by Fraley et al. (2007), polyphosphate kinase has emerged as the most important global regulator of QS system in *P. aeruginosa*. Kornberg et al. (1999) had identified polyphosphate kinase (PPK) enzyme to be important in virulence gene expression, quorum sensing and biofilm formation in

P. aeruginosa. However, the exact mechanism through which PPK regulates QS is still under study.

6.4.9 Two Component Regulatory Systems

6.4.9.1 GacA–GacS

GacA–GacS is the two-component response regulator system where any change in environment acts as a signal for bacteria and specific response is observed. GacA–GacS positively regulates the expression of *lasR* and *rhlR* via controlling the transcription of two small regulatory RNAs (sRNAs), RsmY and RsmZ. These two sRNAs prevent RsmA binding to its mRNA targets (probably *lasR* and *rhlR* genes) and consequently modulate QS directly or indirectly (Brencic et al. 2009). Activity of GacS is counteracted by two inner membrane sensors, RetS and LadS, in response to environmental stimuli that are still unexplored (Ventre et al. 2006). The RetS/LadS/Gac/Rsm cascade is known to be a master regulator of the virulence factors of *P. aeruginosa*, controlling the switch for their expression during the acute/chronic phase transition.

6.4.9.2 PprB

PprB is responsible for positively regulating QS-mediated expression of various virulence factors, viz., proteases, pyocyanin, elastase haemolytic activity and various motilities. Transcriptomics analysis showed the overlapping of genes regulated by both PprB and QS. PprB positively regulates transcription of *lasI*, *rhlI*, *rhlR* and *rsaL*, while it has no effect on *lasR*. PprB also plays important role in maintaining cell membrane permeability and sensitivity to antibiotics (Wang et al. 2003). Till date, the cognate environmental signal for PprB regulator is not known.

6.4.9.3 AlgZR

It is a histidine kinase two-component system which plays an important role in showing two different phenotypes of *P. aeruginosa* (mucoid or nonmucoid). AlgR represses *rhlI* transcription and binds the *rhlI* promoter in vitro directly at the *rhlI*-ABS (5'-CCGTTTCATC-3'), located –28 bp upstream of the transcriptional start site. This leads to reduction of *rhl*-mediated virulence. It also represses the *rhlAB* and *rhlC* encoding rhamnolipids which are important wetting agents for group-coordinated swarming motility (Deziel et al. 2003), needed for structured biofilm formation (Davey et al. 2003; Espinosa-Urgel 2003), and are virulence factors (McClure and Schiller 1992; Jensen et al. 2007).

6.4.9.4 QsrO

It is the most recently discovered QS regulator which is capable to reduce the expression of all three QS systems, i.e. *las*, *rhl* and *pqs*. An ORF 2226 was identified and termed as qsrO (quorum-sensing repressing ORF) in *P. aeruginosa* (Köhler et al. 2014). Further studies are going to decipher its mechanism.

6.5 Quorum-Sensing-Regulated Virulence Factors in *P. aeruginosa*

P. aeruginosa produces a wide range of virulence factors that makes it more infectious for the susceptible host. Most of the virulence factors are QS regulated and are both cell-associated and extracellular products. Among these are lipopolysaccharides (Tang et al. 1996), exotoxin A, exoenzyme S (Nicas et al. 1985), elastase (Woods et al. 1982), lasA (Preston et al. 1997), alkaline protease (Howe and Iglewski 1984), phospholipase C (Vasil et al. 1991), siderophores, viz., pyochelin and pyoverdine (Cox 1982), surface-associated polysaccharide alginate (Pedersen et al. 1992) and pilus-associated adhesions (Tang et al. 1995). Table 6.3 shows different virulence factors and their perspective role in pathogenesis of *P. aeruginosa*.

QS-regulated virulence factors of *P. aeruginosa*

6.5.1 Proteases

The ability of *P. aeruginosa* to invade tissues depends on the production of extracellular enzymes and toxins that break down physical barriers and damage host cells and cause resistance to phagocytosis and the host immune defences. *P. aeruginosa* PAO1 contains about 155 genes encoding proteases. This is about 2.8 % of the whole genome. The basic proteases that have been characterized are elastase A, elastase B, alkaline protease LasA and type IV protease (Caballero et al. 2001). Elastase is a metalloprotease, and its production is regulated by both *lasIR* and *rhlIR* regulons (Whiteley et al. 1999). It degrades the elastin polymer that is widely present in human body. Its concentration is high in cystic fibrosis patients.

Table 6.3 Virulence determinants of pathogenic *P. aeruginosa*

Pathogenic determinants	Virulence factors
Adhesins	Fimbriae (<i>N</i> -methyl-phenylalanine pili) Polysaccharide capsule (glycocalyx) Alginate (biofilm)
Invasins	Elastase Alkaline protease Haemolysins (phospholipase and lecithinase) Cytotoxin (leukocidin) Siderophores and siderophore uptake systems Pyocyanin (diffusible pigment)
Motility/chemotaxis	Retractile pili, flagella
Toxins	Exoenzyme S, exotoxin A, lipopolysaccharide
Antiphagocytic surface properties	Capsule, slime layer, lipopolysaccharides, biofilm formation
Defence against serum bactericidal reaction	Slime layer, capsule, biofilm

The alkaline protease is a zinc-dependent metalloendopeptidase of *P. aeruginosa* and interferes with fibrin formation. Together, elastase and alkaline protease destroy the ground substance of the dermis and other supporting structures composed of fibrin and elastin and enter into the host system. Elastase and alkaline protease together are reported to cause inactivation of gamma interferon (IFN) and tumour necrosis factor (TNF). They are able to degrade IgG and IgA antibodies and help *P. aeruginosa* escape in the immune system (Hoge et al. 2010). Both LasA protease and elastase are secreted with their propeptides. However, whereas LasA protease is exported in its unprocessed proenzyme form, elastase is secreted as a non-covalent complex with its propeptide. The propeptides of both enzymes are degraded extracellularly by the action of elastase and other secreted proteases.

6.5.2 Pyocyanin

P. aeruginosa generates highly diffusible pigmented toxic secondary metabolites, known as phenazines, predominant being the pyocyanin pigment (1-hydroxy-5-methyl-phenazine) which is the key reason for the greenish blue colour of bacteria. Pyocyanin can exist in oxidized or reduced form, the latter being an unstable free radical which reacts rapidly with molecular oxygen. This autoxidation leads to the formation of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) or in the presence of metal catalysts (e.g. iron), the hydroxyl radical ($HO^{\bullet-}$). Although pyocyanin is the major phenazine secreted by *P. aeruginosa*, 1-hydroxyphenazine and phenazine-1-carboxylic acid are also released in lesser quantity (Denning et al. 2003).

Pyocyanin possesses zwitterion property and thus can easily penetrate the cytoplasmic membrane of host cell. Redox cycle involves pyocyanin and NADH of host cell creating highly oxidative environment (generation of reactive oxygen species and H_2O_2). This causes an increase in cytosolic calcium levels, cellular respiration and ATPase expression in human epithelial membrane. Pyocyanin disrupts the beating of human cilia which is beneficial in early-stage establishment of the infection (Wilson et al. 1987). It also inhibits both epidermal cell growth (Cruickshank and Lowbury 1953) and lymphocyte proliferation (Sorenson et al. 1983). Pyocyanin has been recovered from the pulmonary secretions of cystic fibrosis patients infected with *P. aeruginosa* in concentrations that inhibit ciliary beating in vitro. It also has antibiotic activity against a wide variety of microorganisms under aerobic conditions (Hassett et al. 1999), which may benefit *P. aeruginosa* by elimination of other microbes. Pyocyanin also adversely affects RANTES (a chemokine ligand CCL-5) known to attract macrophages, monocytes and eosinophils to the site of inflammation (Denning et al. 1998). The effects of pyocyanin on human immune system have been diagrammatically summarized in Fig. 6.4.

Fig. 6.4 Properties of pyocyanin, a redox pigment secreted by *P. aeruginosa*

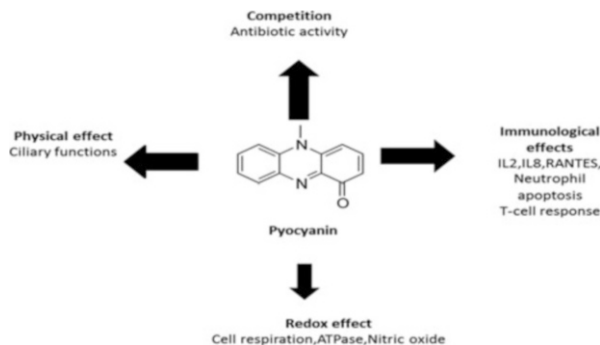
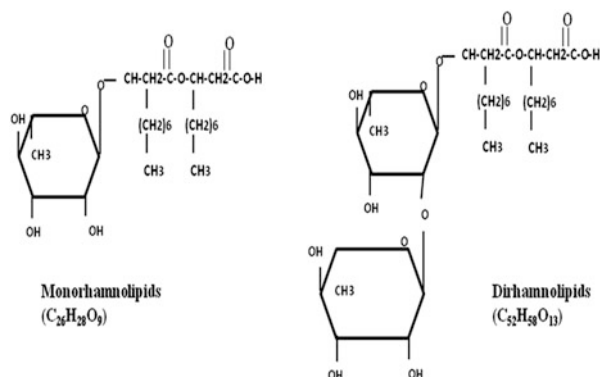


Fig. 6.5 Structure of (a) monorhamnolipids and (b) dirhamnolipids secreted by *P. aeruginosa*



6.5.3 Rhamnolipids

Rhamnolipids are amphipathic molecules that possess tensioactive properties capable of reducing surface tension, forming emulsions and causing pseudosolubilization of insoluble substrates, which allows *P. aeruginosa* to inhabit diverse niches (Caiazza et al. 2005).

Rhamnolipids are comprised of mono- or dirhamnose groups linked to 3-hydroxy fatty acids that vary in length, the most common being L-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (monorhamnolipid) and L-rhamnosyl-L-rhamnosyl-3-hydroxy decanoyl-3-hydroxydecanoate (dirhamnolipid) (Deziel et al. 2003; Lang and Wullbrandt 1999; Maier and Soberón-Chávez 2000) (Fig. 6.5). Rhamnolipids are encoded by operon *rhlAB* induced by *N*-butyryl homoserine lactone-activated RhIR. *rhlA* is involved in the synthesis of precursor and chemotactic signal (3-(3-hydroxyalkanoyloxy) alkanic acids (HAA). *rhlB* encodes first rhamnosyltransferase that catalyses the transfer of TDP-L-rhamnose to HAA molecule leading to the formation of l-rhamnosyl-3-hydroxydecanoyl-3-hydroxydecanoate (monoRL). The second rhamnosyltransferase is encoded by a separate *rhlC* that transfers second rhamnose sugar to monoRL leading to the formation of dirhamnolipids.

Rhamnolipid biosynthesis occurs during the late exponential and stationary phases of growth, typically under conditions of nitrogen or iron limitation. Rhamnolipids are found in the sputa of cystic fibrosis patients and can inactivate tracheal cilia of mammalian cells, indicating that they are virulence factors (Hastie et al. 1986). They are the key modulators of QS-mediated motility of *P. aeruginosa* and maintain mushroom-shaped macrocolonies and open channels in a mature biofilm (Davey et al. 2003). Rhamnolipids assist in biofilm dispersal which aids in dissemination of pathogen to other sites from the focal point (Schooling et al. 2004).

6.5.4 Bacterial Motilities

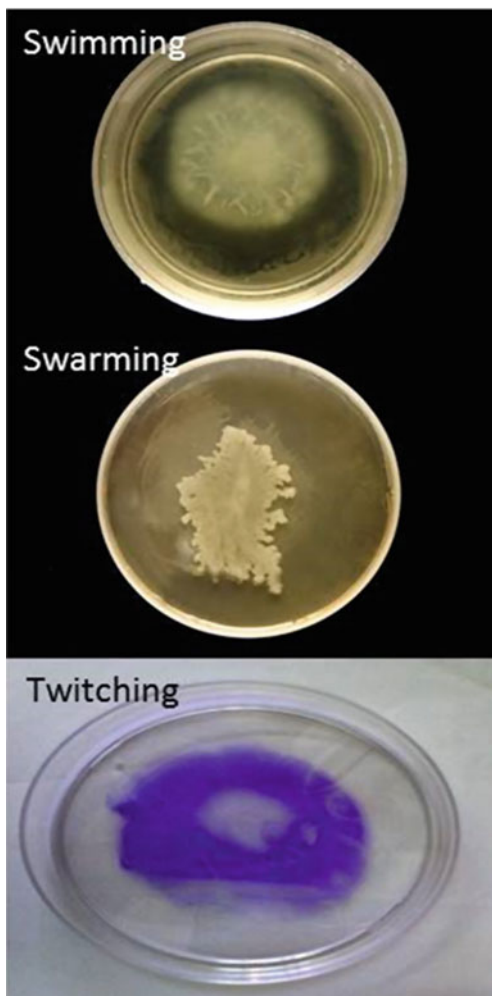
P. aeruginosa is motile in nature and exhibits both flagella-mediated swarming and swimming motilities and pili (specifically type IV)-mediated twitching motility (Fig. 6.6). Motilities provide bacteria to move from infected to naive area so that colonization could be widespread.

The presence of mucosal surfaces at the plausible site of pathogen entry in human host such as that in respiratory, nasal and gastrointestinal tract constantly flushes bacteria away in order to prevent colonization of host mucous membranes. Planktonic *P. aeruginosa* swims across the mucosal surface using its polar flagellum.

Type IV pili and other cell wall adhesions help bacteria attach to the host membrane that activates QS in *P. aeruginosa*. Type IV pili also play a direct role in stabilizing interactions with the abiotic surfaces and/or in the cell-to-cell interactions required to form a microcolony. Type IV pili-mediated twitching motility is necessary for cells to migrate along the surface to form the multicell aggregates. Strains defective in pili biogenesis (like the *pilB* mutant) do not show twitching motility and microcolony formation (O'Toole and Kolter 1998).

Swarming motility shown by *P. aeruginosa* is a multicellular phenomenon involving the coordinated and rapid movement of a bacterial population. Swarming is highly dependent on bacterial cell density, nutrient growth medium and surface moistness (Wang et al. 2004). In addition to physical changes such as increase in the number of flagella or cell elongation, swarmer cell differentiation results in substantial alterations in metabolic bias and gene expression (Tremblay and Deziel 2010), indicating that swarming represents a complex lifestyle adaptation in response to particular medium conditions rather than merely a form of locomotion. Swarming of *P. aeruginosa* is often typified by a dendritic colonial appearance that is dependent on the different concentration ratio of HAA, mono RLs and diRLs (Tremblay et al. 2007). Swarming bacteria are more resistant to antimicrobials and are involved in the formation of biofilm (Overhage et al. 2008).

Fig. 6.6 Motilities exhibited by *P. aeruginosa*



6.5.5 Biofilm

Biofilm is a sessile community characterized by cells that are irreversibly attached to a surface or interface, or to each other, embedded in a matrix of exopolysaccharides and exhibit an altered phenotype with respect to growth rate and gene expression, as compared to planktonic cell (Fig. 6.7). This explanation also extends to the pulmonary infections in cystic fibrosis patients and patients with chronic wounds. It has been estimated that 80 % of microbial infections in the body involve biofilms. Microbes in biofilm are kept together in a biopolymer matrix made of polysaccharides, proteins and DNA from dead cells. This exopolysaccharide (EPS) provides structural stability and protection to biofilm from various antimicrobials due to its impermeable nature. QS is not directly involved in biofilm formation, but

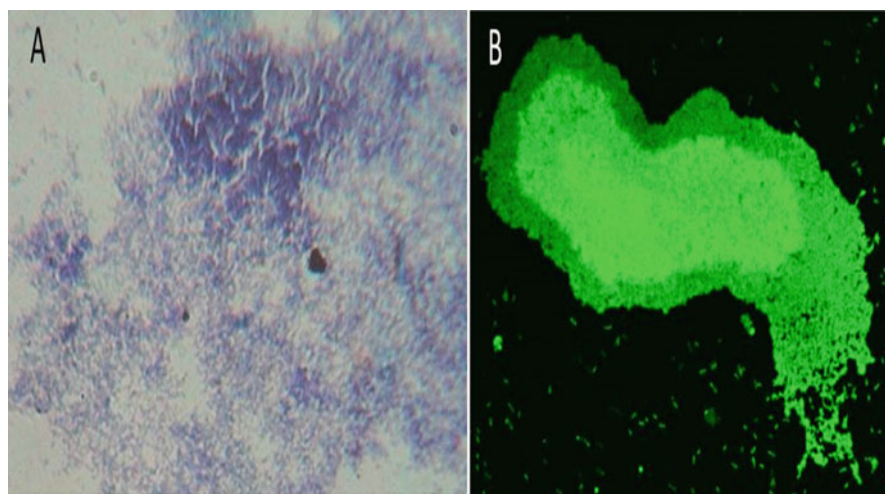


Fig. 6.7 Biofilm formed on glass coverslip and stained with (a) crystal violet and (b) FITC (fluorescein isothiocyanate) observed under light and confocal laser scanning microscope at $\times 10$ magnification, respectively

increased concentration of AHLs in biofilms induces QS-regulated expression of genes in bacterial cell development.

Development of in vitro biofilm is initiated by reversible attachment of planktonic cells to substratum which may be covered by a layer of pellicle (Marshall 1992). At this stage antibiotic sensitivity exists in the culture, and this may be the reason behind the success of perioperative antibiotic prophylaxis. Thereafter, there is irreversible attachment to the surface; bacteria multiply and form microcolonies on surface along with the production of EPS matrix. At this stage, mushroom and stalk-like structures with intervening water channels can be seen, and this exhibits 100–1000 times more tolerance to antibiotic concentration effective against planktonic cells (Fig. 6.7). Davies and colleagues (1998) reported that *las* QS system is essential for creation of mature, differentiated biofilms as indicated by flat and undifferentiated biofilm formed in *las* mutant. Because no discernible differences were observed between the *rhl* mutant and PAO1 wild-type biofilms, it was concluded that the *rhl* QS was not involved (Davies et al. 1998). But in later years, *rhlIR* system was also found to play role in biofilm integration (Hentzer et al. 2001).

The mutation frequency in bacterial cells within biofilm is high and shows multiple drug resistance to different β -lactam antibiotics, aminoglycosides and fluoroquinolones. The presence of exopolysaccharides prevents the entrance of antimicrobials into the bacterial cells embedded in the biofilm matrix.

6.6 Quorum Quenching

Quorum-sensing regulatory system in *P. aeruginosa* is very complex and needs much more study to perfectly lay down the circuit. However, it has been validated that QS system in *P. aeruginosa* modulates itself according to the environment cues and host immune factors to express virulence factors needed for host pathogenesis.

In the last two decades, several compounds (synthetic and natural) have been identified which were capable of inhibiting QS systems in *P. aeruginosa* by interfering with either or all three quorum-sensing systems at the level of (1) signal generation, (2) signal dissemination, (3) signal perception or (4) upstream global regulation, leading to attenuation of virulence, and this is termed as quorum quenching/quorum-sensing inhibition/anti-quorum sensing (Kalia 2013). Interruption of quorum sensing was first demonstrated in *Serratia liquefaciens* which colonizes surface of macro alga *Delisea pulchra*. This colonization was reduced by halo-furanones produced by macro alga which acts as a QS inhibitor. These halo-furanones were ineffective in reducing *P. aeruginosa* virulence. However, C30 and C50 modification of furanone caused increased sensitivity of *P. aeruginosa* biofilm against tobramycin (Hentzer et al. 2003a).

In the search of quorum-sensing inhibitors, various synthetic compounds from chemical libraries were screened and compounds exhibiting remarkable potential were identified. Most remarkable were 3-oxo-C₁₂-(2-aminophenol) (Smith et al. 2003), 4-nitro-pyridine-*N*-oxide (Rasmussen et al. 2005), *N*-(heptyl-sulfanyl acetyl)-L-HSL (HepS-AHL) (Persson et al. 2005) and acyl cyclopentylamines (Geske et al. 2007) which significantly reduced QS-regulated virulence in *P. aeruginosa*.

Several medicinal plants also showed QS inhibitory potential which were considered safer in terms of toxicity, in comparison to the synthetic compounds. Extracts of plants like *Allium sativum* (Bjarnsholt et al. 2005), *Cinnamomum zeylanicum* (Niu et al. 2006), *Brassica oleracea* and *Vitis vinifera* (Vattem et al. 2007), *Ananas comosus* and *Ocimum sanctum* (Musthafa et al. 2010), *Combretum albiflorum* (Vandeputte et al. 2010) and *Terminalia chebula* (Sarabhai et al. 2013) cause inhibition of QS-regulated virulence gene expression in bacteria. Adonizio et al. (2008) studied extracts of *Conocarpus erectus*, *Chamaesyce hypericifolia*, *Callistemon viminalis*, *Bucida buceras*, *Tetrazygia bicolor* and *Quercus virginiana* for quenching quorum sensing in *P. aeruginosa*. Many phytochemicals were also identified as quenchers of quorum sensing in *P. aeruginosa*, viz., epigallocatechin, ellagic acid (Huber et al. 2003), vanillin (Choo et al. 2006), cinnamaldehyde (Niu et al. 2006), castalagin and vescalagin (Adonizio et al. 2008) and caffeine (Norizan et al. 2013).

Quorum-sensing inhibitors also enhance the effectivity of antibiotics in treating *P. aeruginosa* infections. The effectiveness of tobramycin (antibiotic) increased when used along with baicalin hydrate, and load of *Burkholderia cenocepacia* population in the lungs of infected mouse model was reduced (Brackman et al. 2011). Vancomycin in association with hamamelitannin also enhanced the survival of bacteria-infected *C. elegans* and *Galleria mellonella* in comparison to

vancomycin alone or either of the two compounds used singly (Brackman et al. 2011).

In the conventional treatment of opportunistic infections with antibiotics presents a selection pressure where resistant strains survive and are maintained in the population. In contrast, quorum-sensing inhibitors reduce the cell density-dependent virulence factors and attenuate pathogens. As QS inhibitors do not act on the vital genes of bacteria, they exhibit low risk of emergence of resistance mutants in the population. Hence, better understanding of QS systems in notorious pathogens will give us a way to combat diseases by an alternative therapy based on QS inhibition.

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In Silico Comparative Analysis of Type VI Secretion Systems in *Pseudomonas putida* LS46

7

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Contents

7.1	Introduction	258
7.2	Type VI Secretion Systems (T6SS) in Bacteria	258
7.3	Identification of T6SS Components in <i>Pseudomonas putida</i> Strains	260
7.4	Comparison of T6SS of <i>P. putida</i> LS46 with <i>P. aeruginosa</i> , <i>V. cholerae</i> , and <i>R. leguminosarum</i>	262
7.5	Identification of T6SS in Other <i>P. putida</i> Strains	265
7.6	Homology of T6SS Proteins of <i>P. putida</i> LS46 with Other T6SS Proteins	266
7.7	Phylogenetic Analyses of T6SS Proteins in <i>P. putida</i> Strains	266
7.8	Role of T6SS Effector Proteins in Bacteria	270
7.9	Polyhydroxyalkanoate Synthesis in <i>P. putida</i> LS46	271
7.10	Regulation of PHA Synthesis	272
7.11	Crosstalk Communication Among Quorum Sensing, T6SS, and PHA Synthesis	273
7.12	Conclusions	274
	References	275

Abstract

Six secretion systems (Type I–Type VI) have been reported in Gram-negative bacteria. The Type VI secretion (T6SS) system was identified for the first time in two human pathogens, and its components assemble a system for secretion of the hemolysin-coregulated protein (Hcp1) and the valine glycine repeat protein (VgrG). Putative genes encoding T6SS are present not only in bacteria identified as human or plant pathogens but also in symbiotic nitrogen-fixing bacteria and nonpathogenic soil bacteria. *Pseudomonas putida* LS46 was isolated from wastewater and was identified as a producer of medium chain length

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polyhydroxyalkanoates (mcl-PHAs), natural polyester polymers synthesized by bacteria as energy storage molecules. Using the COG ID of the T6SS from *P. aeruginosa* PAO1 as a probe, three putative T6SSs were identified in *P. putida* LS46. Two of the T6SSs (S1 and S2) were identical in their organization, and their gene products showed a high degree of amino acid sequence homology. The third T6SS (S3) had a different gene organization, and some of the T6SS genes present in S1/S2 were absent in S3. Many *P. putida* strains have highly conserved T6SS genes, which have low amino acid sequence identity to T6SS proteins of *Vibrio cholerae*, *Aeromonas hydrophila*, *Rhizobium leguminosarum*, and *Burkholderia mallei*. Accumulation of PHA in bacteria helps to withstand starvation and survive under hostile environmental conditions. We speculate the GacA/GacS system regulates Quorum Sensing, which in turn regulates T6SS. T6SS effector molecules could be involved in signal transduction within the population for induction of PHA synthesis and accumulation.

7.1 Introduction

Interspecies bacterial interactions are important for ecosystem functioning. Bacteria interact with their environments (i.e., water, soil, or specific host tissues) within, and often between, different communities by activating secretion systems. Gram-negative bacteria are known to excrete a range of molecules, from small effector proteins to multi-component molecular complexes, to communicate within microbial communities, to stimulate the growth of their own populations, and/or to antagonize rival species. So far seven secretion systems (Type I–Type VII) have been identified in Gram-negative bacteria. These secretion systems regulate the interactions between bacteria and their environments by delivering extracellular proteins through secretion or injection. Type III (T3SS), Type IV (T4SS), and Type VI (T6SS) secretion systems have received considerable attention because they assemble a specialized needle-like apparatus to inject effector molecules directly into host cells (Alvarez-Martinez and Christie 2009; Cornelis 2006; Filloux et al. 2008).

7.2 Type VI Secretion Systems (T6SS) in Bacteria

The Type VI secretion system (T6SS) was first identified in two pathogenic bacteria, *Pseudomonas aeruginosa* PAO1 and *Vibrio cholerae* ATCC39315 (Pukatzki et al. 2006; Mougous et al. 2006). In *Vibrio cholerae*, secretion of hemolysin-coregulated protein (Hcp) and valine–glycine repeat protein G (VgrG) was mediated via the IcmF-associated homologous protein gene cluster that is essential for cytotoxicity of *V. cholerae* toward *Dictyostelium amoebae* (Pukatzki

et al. 2006). However, structural data of the *V. cholerae* T6SS provided evidence of its role in patients with cystic fibrosis with chronic lung infection due to *Pseudomonas aeruginosa* (Mougous et al. 2006). This system was further identified in other pathogens, like *Burkholderia pseudomallei* (Burtnick et al. 2011), *Edwardsiella tarda* (Rao et al. 2004), *Francisella tularensis* (Nano et al. 2004), *Helicobacter hepaticus* (Bartonickova et al. 2013), and *Campylobacter jejuni* (Lertpiriyapong et al. 2012).

Although the initial investigations of T6SS were associated with pathogenesis (Bröms et al. 2012; Burtnick et al. 2011; Suarez et al. 2008), recent studies showed that T6SS could also promote commensal or mutualistic relationships between bacteria and eukaryotic hosts, as well as mediate cooperation or competition between bacteria (Decoin et al. 2014; Jani and Cotter 2010; Tashiro et al. 2013). Type VI Secretion Systems were present in symbiotic nitrogen-fixing bacteria and nonpathogenic bacteria, and thus, it appears that T6SSs may be involved in functions other than virulence.

The components of T6SSs were identified as early as 2003 in *Vibrio cholera* and *Rhizobium leguminosarum* (Das and Chaudhuri 2003; Bladergroen et al. 2003). Mutation analyses of *R. leguminosarum* identified a cluster of 14 genes that were shown to be involved in nodulation in host plant roots. These loci were designated as “impaired in nodulation” (*imp*) mutations. Two *R. leguminosarum* proteins, ImpK and ImpL, had high amino acid sequence identities with DotU and IcmF proteins, which are components of the Type VI secretion system.

Das and Chaudhuri (2003) identified another secretion system in *V. cholerae* containing homologues of the DotU and IcmF proteins. Transposon mutagenesis of *V. cholerae* identified insertions in the region designated as the “virulence-associated secretion T6SS region” (*vas*), and some of the *vas* genes had high amino acid identity to the *imp* gene products encoded by *R. leguminosarum*. Further, it was established that this system was responsible for secretion of the Hcp (hemolysin-coregulated protein) and VgrG (valine glycine rich) proteins, and that secretion of the VgrG was dependent on the presence of the Hcp protein. These initial observations led to the identification of T6SSs in *P. aeruginosa* PAO1 and *V. cholerae* V52 in 2006 (Pukatzki et al. 2006; Mougous et al. 2006).

T6SS proteins (including Hcp) are widespread in Proteobacteria, particularly among gamma-Proteobacteria (Bonemann et al. 2010; Shrivastava and Mande 2008). Multiple copies of T6SS sequences with complete loci is a common feature among pathogenic and nonpathogenic bacteria. Comparative genomic analyses suggest that T6SS sequences were acquired by pathogenic and nonpathogenic bacteria via horizontal gene transfer, and the role of genomic islands observed in different bacteria have been implicated in this horizontal gene transfer (Miyata et al. 2013). The T6SSs consist of membrane-associated assemblies, with two proteins that are homologous to T4SS proteins, and another assembly resembling the bacteriophage T4 sheath, tube, and tail spike proteins. These two assemblies work together to transport the effector proteins across the envelope of the donor cell and then through the outer membrane of a recipient cell.

Type VI Secretion Systems have been identified in 25 % of all Gram-negative bacteria sequenced to date, but only the T6SSs of *V. cholerae* and *P. aeruginosa* have been studied in detail. Type VI Secretion Systems in *P. aeruginosa* PAO1 consist of 13 conserved genes (Boyer et al. 2009; Zheng and Leung 2007). Silverman et al. (2012) classified these gene products on the basis of their functions and identified three functional components. Functional component 1 consists of three proteins, TssL (IcmF, COG3523), TssM (DotU, COG3455), and TssJ (COG3521), which are membrane-bound proteins that interact to form a complex that spans bacterial cell walls. Functional component 2 consists of the Hcp protein (COG3157), which is similar to the major tail protein (gpV) of bacteriophage λ . Hexamers of the Hcp, VgrG (COG3501), TssB (COG3517), and TssC (COG3518) proteins are involved in the formation of tubular structure with a diameter of 400 nm. VgrG is related to the gp27/gp5 proteins of bacteriophage T4 and is associated with the formation of spike-like structures similar to the bacteriophage T4 tail (Leiman et al. 2009). Two components, the TssB/TssC subunits, are highly similar to the gp18 protein of bacteriophage T4 and form a tubular structure similar to the sheath of phage T4. Contraction of this sheath ejects the tube protein and punctures the cell wall of target cell, resulting in the injection of the effector molecule (Bonemann et al. 2010). Functional component 3 consists of the TssE (COG3520), ClpV (COG 0542), TssB (COG3516), TssC (COG3517), IcmH (COG3455), and IcmF (COG3523) proteins. TssE is related to the bacteriophage T4 base-plate protein (gp25). The ClpV protein is an ATPase that provides the energy for the contraction of the TssB/TssC heterodimer. IcmF is an intermembrane protein with ATPase activity required for the structural components of T6SS and binds to IcmH to form a transmembrane complex.

7.3 Identification of T6SS Components in *Pseudomonas putida* Strains

P. putida LS46 was isolated from wastewater as a bacterium that synthesizes medium chain length polyhydroxyalkanoate (mcl-PHA) polymers (Sharma et al. 2012). Nucleotide sequence analysis of the *P. putida* LS46 genome revealed 86.5–97.2 % nucleotide sequence identity to 10 other *P. putida* strains isolated from different geographical locations and ecological niches (Sharma et al. 2013, 2014). *P. putida* KT2440 and *P. putida* BIRD1 were isolated from rhizosphere soil and developed as plant growth-promoting rhizobacteria (Matilla et al. 2011; Nakazawa 2002). *P. putida* F1 was isolated from polluted soil and developed as a bioremediation agent (Choi et al. 2003; Eaton 1997). *P. putida* S16 was isolated in China and identified as a nicotine-degrading strain (Tang et al. 2012), while *P. putida* W619 was isolated as an endophyte of poplar (Taghavi et al. 2005). *P. putida* GB1 was isolated from fresh water and identified as manganese oxidizer (Wu et al. 2011). 16S rDNA and *cpn60* gene sequencing confirmed the close phylogenetic relationship among these *P. putida* strains (Sharma et al. 2014).

Boyer et al. (2009) identified T6SS genes in various Gram-negative bacteria, including *P. putida*, using comparative *in silico* genome analyses. Components of a T6SS were also identified in a *Pseudomonas* sp. by Barret et al. (2011), and 16 COGs have been listed as components of T6SS. These COGs were used to search homologous T6SS sequences in the *P. putida* LS46 genome. More specifically, BLASTp analysis using amino acid sequences of *P. aeruginosa* PAO1 T6SS proteins (different COGs) to query the *P. putida* LS46 genome revealed three putative T6SS gene clusters, designated T6SS clusters S1, S2, and S3. The GC ratios of the S1, S2, and S3 T6SS clusters in *P. putida* LS46 were different. The *P. putida* LS46 S1, S2, and S3 clusters had 58.1 %, 61.7 %, and 63.1 % GC, respectively. Among the T6SS clusters of *P. putida* LS46, only S2 had the same GC ratio as the *P. putida* LS46 genome (61.7 %). DNA sequences with GC ratios that differ from the GC ratio of the majority of genome sequence are indicative of horizontal gene transfer (Miyata et al. 2013).

All the three of the putative *P. putida* LS46 T6SS gene clusters encoded Hcp1, VgrG, and DotU proteins. The S1 and S2 clusters encoded two Fha-domain

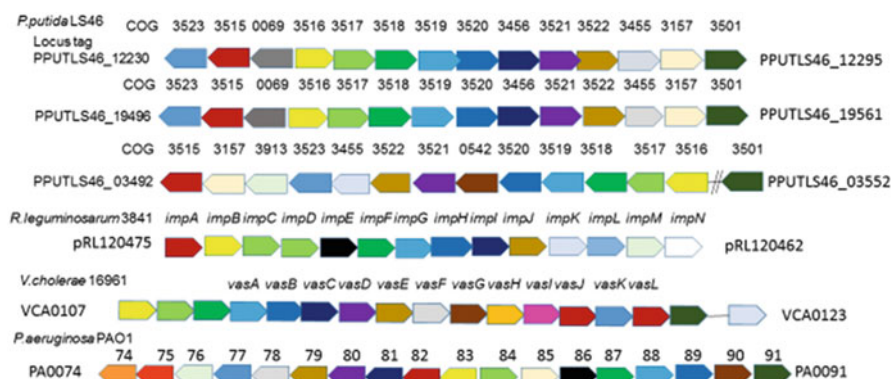


Fig. 7.1 Organization of T6SSs in *P. putida* LS46 and comparison to T6SSs of *Pseudomonas aeruginosa* PAO1, *Vibrio cholerae* 16961, and *R. leguminosarum* 3841. COG families are color-coded, and the same colors represent the same COGs

containing proteins (PPUTLS46_12270, PPUTLS46_19536) and two glutamate synthetase-domain proteins (PPUTLS46_12270 and PPUTLS46_19536). The S3 cluster encoded three genes not present in clusters S1 and S2: PPUTLS46_03502 (COG3913) and PPUTLS46_03527 (COG0542). PPUTLS46_03527 encodes the ClpB protein, which is an ATPase that provides energy for contraction of the sheath. The proteins encoded by the S1 and S2 clusters had relatively high amino acid identities (64–100 %), whereas proteins of S3 cluster had low amino acid identities (27–38 %) to their corresponding S1 and S2 proteins. In addition to the three VgrG proteins (one each encoded in clusters S1, S2, and S3), two additional “orphaned” VgrG proteins (PPUTLS46_02652 and PPUTLS46_09384) were identified, and these had high amino acid sequence identities (73 %) with the VgrG protein of cluster S1 and S2, but very low amino acid sequence identity (30 %) with the VgrG protein of cluster S3.

7.4 Comparison of T6SS of *P. putida* LS46 with *P. aeruginosa*, *V. cholerae*, and *R. leguminosarum*

The majority of T6SS components present in *P. putida* LS46 were homologous to T6SS genes in *P. aeruginosa*, *V. cholerae*, and *R. leguminosarum*. However, some of the genes encoding T6SS proteins in *P. aeruginosa*, *V. cholerae*, or *R. leguminosarum* were absent in the T6SS clusters of *P. putida* LS46. For example, two genes encoding T6SS proteins (ImpE and ImpN) in *R. leguminosarum* were absent in *P. putida* LS46 genome. The ImpE protein (COG 4455) of *R. leguminosarum* was classified as a virulence factor involved in temperature-dependent secretion, while the ImpN protein was identified as a serine threonine kinase (STK) involved in nitrogen fixation (Bladergroen et al. 2003).

The T6SS cluster HSI-1 of *P. aeruginosa* encodes two STK genes: ppkA (COG 0515) and phosphatase pppA (COG 0631) (Ho et al. 2014). These proteins have antagonistic role in Hcp secretion. The *P. putida* LS46 genome encodes five STK homologues that are classified in different COG Families. A homolog of the *P. aeruginosa* pppA protein was identified in the *P. putida* LS46 genome, but a homolog of the *P. aeruginosa* ppkA protein was absent (Table 7.1).

Bingle et al. (2008) studied the evolutionary relationships of different T6SS in bacteria on the basis of the DUF770 (COG 3516) and DUF877 (COG 3517) proteins. T6SSs were classified into four categories: A, B, C, and D. *Pseudomonas aeruginosa* PAO1 secretion systems HSI-I, HSI-II, and HSI-III were placed in groups A, D, and B, respectively. The *P. putida* LS46 T6SS secretion system is homologous to the *P. aeruginosa* PAO1 T6SS cluster HSI-III and therefore can be placed in Group B. Further analyses of 11 core T6SS proteins in 34 *Pseudomonas* species by Barret et al. (2011) identified 5 groups: 1, 2, 3, 4A, and 4B among *Pseudomonas* species. Cluster 4A included *P. aeruginosa* and *P. fluorescens* strains, while *P. putida* clusters S1, S2, and S3 were placed in groups 1, 2, and 4B, respectively. The *P. putida* LS46 T6SS clusters S1 and S2 were identical to the T6SS cluster HSI-I of *P. aeruginosa* PAO1, while the *P. putida*

Table 7.1 Homology of T6SS (S1 and S2) proteins of *P. putida* LS46 with *P. aeruginosa*, *V. cholerae*, *R. leguminosarum*, and other *P. putida* strains

	LS46	PAOI	N16961	3841	KT2440	F1	GB-1	W619	S16
COG	PputLS46_	PA_	VC_/VCA	pRL_120	PP_	Pput_	PputGB1_	PputW61_	PPS_
3501	12290	0091 (37)	0123 (39)	480 (32)	2614 (91)	2117 (82)	3234 (71)	2517 (29)	2828 (28)
	19561 (100)	0095 (32)	0018 (39)		4049 (71)	2618 (28)	2760 (28)	3254 (31)	2864 (66)
	03652 (30)	2373 (33)			3106 (30)				2168 (74)
	12290	5267 (53)	0017 (62)	–	2615 (100)	2118 (97)	3233 (75)	3256 (33)	–
3157	19556 (100)	1512 (53)	1415 (67)		4073 (100)		2776 (27)		
	03497 (34)	0263 (53)			3233 (75)				
	12285	1668 (45)	0115 (42)	465 (24)	4081 (93)	2372 (68)	3232 (90)	2502 (26)	2844 (27)
3455	19551 (85)	0078 (34)			2616 (82)	2119 (81)	2772 (28)		
	3517 (22)				3092 (26)	2630 (26)			
	12280	0079 (45)	0114 (45)	466 (35)	4080 (93)	2120 (91)	3231 (90)	2503 (24)	2843 (26)
3522	19346 (90)	1667 (35)			2617 (91)	2629 (26)	2771 (26)	3259 (28)	2166 (65)
	3522 (26)				3093 (26)				
	12275	0080 (29)	0113 (32)	–	4079 (97)	2121 (76)	3230 (75)	–	–
3521	19541 (76)				2618 (75)				
	3527				3094 (24)				
	12270	–	–	–	2619 (64)	2122 (64)	3239 (64)	–	–
3456	19536 (64)								
	3532								
	12265		0111 (44)	468 (26)	4078 (97)	2123 (88)	3228 (90)	3244 (28)	2840 (27)
3520	19531 (85)				2620 (88)	2626 (26)	2768 (27)	2506 (28)	
	3537 (27)				3096 (26)				
	12260	1660 (32)	0110 (47)	469 (29)	4077 (99)	2124 (86)	3227 (86)	2507 (30)	2839 (30)
3519	19526 (86)	0088 (28)			2621 (86)	2625 (30)	2767 (30)	3245 (28)	
	3542 (31)	2369 (29)			3097 (30)				

(continued)

Table 7.1 (continued)

COG	LS46	PAO1	N16961	3841	KT2440	F1	GB-1	W619	S16
	PputLS46_	PA_	VC_/VCA	pRL_120	PP_	Pput_	PputGB1_	PputW61_	PPS_
3518	12255	1659 (29)	0109 (36)		4076 (06)	2125 (85)	3226 (81)		
	19521 (85)				2622 (85)				
3517	12250	1658 (65)	0108 (68)	472 (39)	2623 (92)	2126 (92)	3225 (91)	3260 (41)	2837 (37)
	19516 (92)	0084 (41)			3099 (37)	2623 (37)	2765 (37)	2509 (37)	
	3552	2366 (39)							
	12245	1657 (50)	0107 (55)	474 (30)	4074 (97)	2127 (82)	3224 (79)	3261 (36)	2836 (33)
3516	19511 (83)	0083 (33)			2624 (83)	2622 (33)	2764 (33)	2510 (33)	
	3557 (34)	2365 (30)			3100 (33)				
	12240	–	0118 (29)	–	4073 (98)	2128 (64)	3223 (61)	–	–
	19506 (65)				2625 (64)				
3515	12235	1656 (25)	0119 (23)	–	4072 (96)	2129 (77)	3222 (78)	3246 (22)	
	19501 (78)		120 (38)		2626 (78)				
3523	12230	0077 (26)	0129 (36)	464 (21)	4071 (96)	2130 (83)	3221 (80)	2499 (27)	2847 (27)
	19496 (83)	1669 (25)			2627 (83)	2633 (25)	2775 (27)	3247 (23)	2845 (27)
	3562 (26)				3090 (25)	2631 (26)	2773 (28)		

Figures in parenthesis indicate % homology to *P. putida* LS46 proteins

LS46 T6SS S3 was identical to the HSI-III cluster of *P. aeruginosa* PAO1 and was placed in group 4B, which was a sub-clade of group 4A which contains the *P. aeruginosa* PAO1 HSI-III cluster.

7.5 Identification of T6SS in Other *P. putida* Strains

Type VI Secretion System proteins of *P. putida* LS46, classified in 16 COG families, were also present in other *P. putida* strains, including *P. putida* KT2440, F1, W619, GB-1, and S16. Homologs of all three T6SS clusters (S1, S2, and S3) were identified in *P. putida* KT2440, while in strains F1, W619, and GB-1 contained two only T6SS clusters. Only one T6SS was detected in *P. putida* strain S16, and all the three T6SSs sequences were absent in *P. putida* strain BIRD1 (a soil-isolate from Spain).

The organization of the genes in the three T6SS clusters of *P. putida* strains is shown in Fig. 7.2. As indicated above, the T6SS S1 and S2 clusters in *P. putida* LS46 had identical organizations and were equivalent to the HSI-I cluster of *P. aeruginosa* PAO1, while the organization of the *P. putida* LS46 cluster S3 was different from those of clusters S1 and S2 and identical to the *P. aeruginosa* PAO1 cluster 4B. In other *P. putida* strains, the T6SS clusters had similar organizations to either cluster S1 or cluster S2. The ClpB (COG 0542) protein (ATPase) was present in cluster S3, but not in clusters S1 or S2. Another difference between T6SS clusters

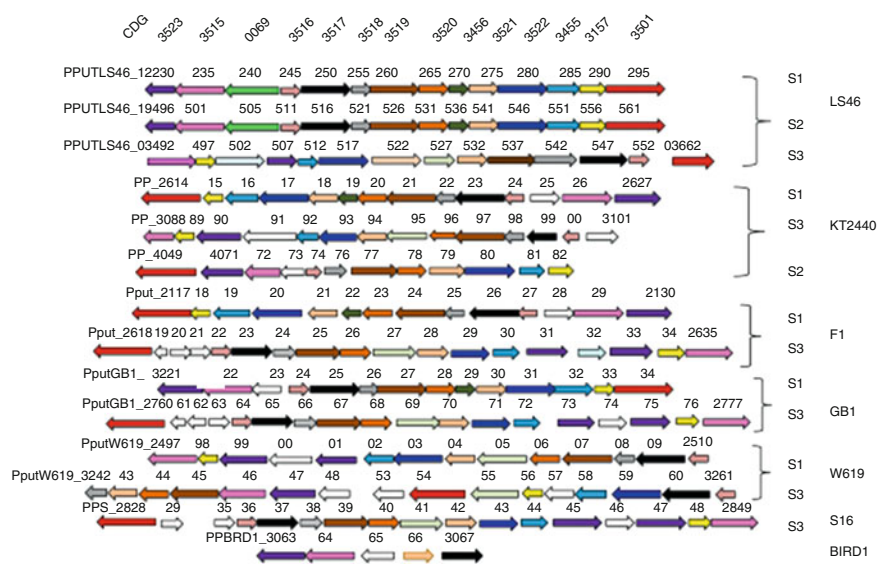


Fig. 7.2 T6SSs of different *P. putida* strains. COG families are color-coded, and the same colors represent the same COGs. Genes are not drawn to scale. *White color* shows genes that are not related to T6SS

in different *P. putida* strains was the presence and location of genes that encode the ImpM protein (COG 3913). ImpM, a homolog of the BMA_A0400 family, was present in the S3 clusters of *P. putida* LS46, *P. putida* F1, and *P. putida* W619, but in the S1 clusters of *P. putida* S16.

7.6 Homology of T6SS Proteins of *P. putida* LS46 with Other T6SS Proteins

The amino acid sequences of *P. putida* LS46 T6SS proteins were compared with six *P. putida* strains, two pathogenic bacteria (*P. aeruginosa* and *V. cholera*), and one symbiotic bacterium (*R. leguminosarum*) using BLASTp. Only BLASTp E-values < 1e-5 were considered significant. Although the gene organization of the *P. putida* LS46 T6SS cluster S1 and cluster S2 were identical, the amino acid sequences of homologous proteins were dissimilar. The proteins of T6SS clusters S1 and S2 in different *P. putida* strains had amino acid identities that ranged from 65 to 100 % (Table 7.1). In contrast, amino acid sequence identities of cluster S3 proteins were very low (22–30 %) compared with corresponding proteins in clusters S1 and S2 (Table 7.2).

The amino acid sequences of *P. putida* LS46 T6SS cluster S1 and S2 proteins had low levels of identity with corresponding T6SS proteins of *P. putida* strains W619 and S16, *P. aeruginosa*, *V. cholerae*, and *R. leguminosarum* (Tables 7.1 and 7.2). These ranged from 32 to 65 % identity between *P. putida* strains and *P. aeruginosa*, 32–68 % identity between *P. putida* strains and *V. cholera*, and 21–39 % identity between *P. putida* strains and *R. leguminosarum*. The T6SS protein in clusters S1, S2, and S3 of *P. putida* LS46 also had low amino acid sequence identities with corresponding proteins of the *P. aeruginosa* clusters HSI-I, HSI-II, and HSI-III.

In contrast, the amino acid sequences of T6SS cluster 3 proteins were highly conserved among *P. putida* strains. Proteins of *P. putida* LS46 T6SS cluster S3 had higher amino acid identities (65–99 %) with corresponding T6SS proteins of *P. putida* strains KT2440, F1, GB1, W619, and S16 (Tables 7.1 and 7.2).

7.7 Phylogenetic Analyses of T6SS Proteins in *P. putida* Strains

The diversity of T6SS sequences in bacteria was initially studied on the basis of concatenated amino acid sequence of DUF770 and DUF887 proteins (Bingle et al. 2008) or based on 11 core T6SS genes present in all *Pseudomonas* species (Barret et al. 2011). The analysis conducted by Barret et al. (2011), however, did not include *P. putida* LS46 and *P. putida* S16. In present study, we used the amino acid sequences of the IcmF and DotU proteins to establish the diversity and evolutionary relationships among the three T6SS clusters in *P. putida* strains, as

Table 7.2 Homology of T6SS (S3) proteins of *P. putida* LS46 with *P. aeruginosa*, *V. cholerae*, *R. leguminosarum*, and other *P. putida* strains

PputLS46	COG	PA_	VC_VCA_	pRL120	PP_	Pput_	PputGB1_	PputW619	PPS_
03662	3501	0095 (40)	0018 (32)	480 (32)	3106 (99)	2618 (99)	2760 (65)	2577 (65)	2828 (76)
03557	3516	1657 (33)	0107 (37)	474 (52)	3100 (99)	2622 (100)	2764 (98)	2510 (94)	2836 (99)
		0083 (42)							
		2365 (61)							
03552	3517	1658 (38)	0108 (38)	472 (35)	3099 (94)	2623 (94)	2765 (93)	2509 (92)	2837 (94)
		0084 (47)							
		2366 (54)							
03547	3518	0087 (30)	–	470 (27)	3098 (90)	2624 (99)	2767 (89)	2508 (87)	2838 (88)
03542	3519	1660 (32)	0110 (34)	469 (27)	3097 (100)	2625 (99)	2768 (95)	2507 (94)	2839 (96)
		0088 (34)							
		2369 (33)							
03537	3520	0089 (32)	0111 (26)	468 (32)	3096 (100)	2626 (99)	3228 (28)	2506 (94)	2840 (97)
		1661 (29)							
		2370 (29)							
03532	0542	2371 (53)	0116 (45)	279 (55)	3095 (87)	2627 (99)	2769 (85)	2505 (95)	2841 (96)
		0090 (48)		213 (48)					
		1662 (53)							
03527	3521	0080 (29)	0113 (27)	–	3094 (99)	2628 (99)	2770 (93)	2504 (79)	2842 (92)
		1666 (33)							
03522	3522	0079 (27)	VCA0114 (24)	466 (28)	3093 (91)	2629 (91)	2772 (96)	2503 (98)	2843 (97)
		2363 (27)							
03517	3455	1668 (34)	0115 (25)	–	3092 (99)	2630 (99)	3232 (23)	2502 (93)	2844 (95)
						2372 (24)	2558 (23)		
03512	3523	0077 (35)	0120 (27)	464 (31)	3091 (90)	2631 (90)	2773 (93)		
		1669 (33)				2633 (30)		2501 (89)	2845 (85)
03507	3913	–	–	463 (34)	–	2632 (96)	2774 (83)	2500 (77)	2846 (81)

(continued)

Table 7.2 (continued)

PputLS46	COG	PA_	VC_VCA_	pRL120	PP_	Pput_	PputGB1_	PputW619	PPS_
03502	3523	0077 (35)	0120 (27)	464 (31)	3090 (90)	2631 (99)	2773 (94)	2501 (89)	2847 (55)
		1669 (33)				2633 (30)	2775 (31)		
03497	3157	0085 (29)	–	477 (24)	3089 (99)	2634 (99)	2776 (98)	2498 (93)	2848 (87)
		2367 (26)							
03492	3515	2360 (31)	–	475 (23)	3088 (99)	2635 (99)	2777 (91)	2497 (83)	2849 (89)

Figures in parenthesis indicate % homology to *P. putida* LS46 proteins

well as the pathogenic strains *P. aeruginosa* PAO1 and *V. cholerae*, and the symbiotic nitrogen fixer, *R. leguminosarum*.

Phylogenetic analyses with the T6SS cluster S1 protein IcmF resulted in a clade that contained *P. putida* LS46, *P. putida* KT2440, *P. putida* F1, and *P. putida* GB1. The IcmF proteins of cluster S2 from *P. putida* LS46 and *P. putida* KT2440 were also present in this clade. The IcmF proteins of cluster S3 were present in all six *P. putida* strains, and phylogenetic analyses of these proteins revealed a clade consisting of *P. putida* LS46, *P. putida* KT2440, *P. putida* F1, *P. putida* S16, *P. putida* GB1, and *P. putida* W619. However, a second IcmF protein from *P. putida* W619, which was thought to be component of the S1 cluster, was different from the IcmF proteins of all the other *P. putida* strains investigated. The IcmF proteins of *P. aeruginosa* PAO1 and *R. leguminosarum* 3841 formed a clade that was separate and different than IcmF protein of the *P. putida* strains. The IcmF protein of *V. cholerae* was related to the IcmF proteins of the *P. putida* strains and was present in the same cluster. Similar clustering was observed on the basis of DotU, which is another component of T6SS in different bacteria. DotU proteins of S1 and S2 of *P. putida* strains LS46, F1, KT2440, GB1 clustered together, but DotU proteins from S3 of *P. putida* strains formed a separate clade (Fig. 7.3).

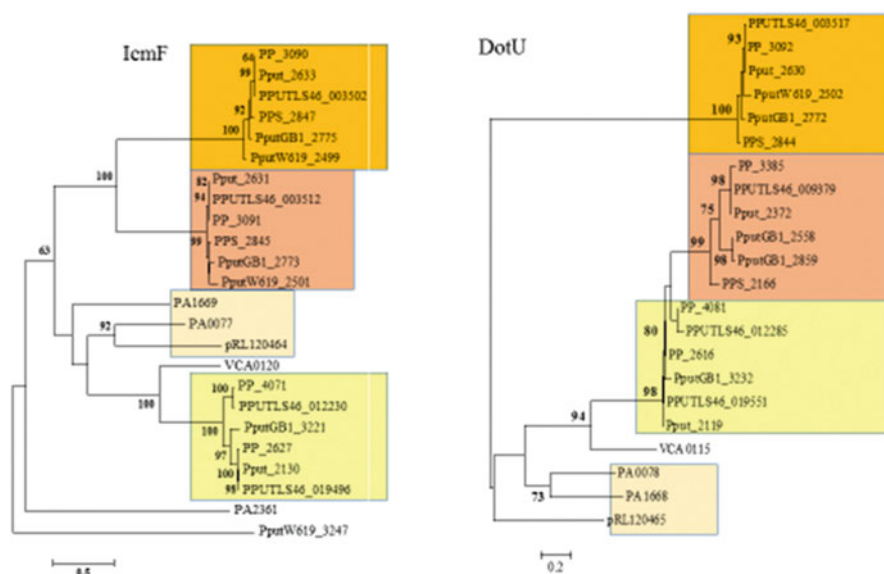


Fig. 7.3 Phylogeny of IcmF and DotU proteins of different T6SSs of *P. putida* strains, *P. aeruginosa*, *V. cholerae*, and *R. leguminosarum*. Protein sequences of IcmF (COG 3523) and DotU (COG 3455) were aligned using Clustal W, and phylogenetic trees were constructed with Molecular Evolutionary Genetics Analysis (MEGA) version 5.0.3 using neighbor joining method with Poisson correction, complete gap deletion, and bootstrapping ($n = 1000$) parameters

7.8 Role of T6SS Effector Proteins in Bacteria

The role of T6SS in plant and animal pathogens has been well documented (Burtnick et al. 2011; Russell et al. 2011; Bartonickova et al. 2013; Ho et al. 2014; Altindis et al. 2015). The effector molecules, their role in pathogenicity, and the mechanisms of bacterial–host interactions have been reviewed by Russell et al. (2014). Multiple T6SSs are regulated differentially under different environmental conditions and release different effector molecules (Altindis et al. 2015; Sana et al. 2012; Sheng et al. 2012). Three effectors molecules with different functions in pathogenicity were identified in *P. aeruginosa* PAO1, and their roles have been well documented (Russell et al. 2014). In other pathogens, like *Salmonella enterica* and *Helicobacter hepaticus*, T6SS suppressed virulence so that the bacteria inside the host were able to multiply better (Bartonickova et al. 2013). In other pathogens, like *Yersinia pestis*, inactivation of T6SS led to greater proliferation inside host cells with increased fitness (Russell et al. 2014). In *R. leguminosarum*, mutations in T6SS conferred the ability to nodulate in non-host plants (Bladergroen et al. 2003).

Tse2, a secreted effector of *P. aeruginosa* PAO1, is an antibacterial, while Tse1 and Tse3 are lytic enzymes acting on peptidoglycan of bacteria (Hood et al. 2010). Tse2 was shown to inhibit the growth of other bacteria and provided a pronounced fitness advantage for *P. aeruginosa* (Li et al. 2012). *P. aeruginosa* and *P. putida* coexist in nature and compete for ecological niches. In mutant strains of *P. aeruginosa* where *tse1* and *tse3* were deleted, *P. aeruginosa* was unable to compete with *P. putida* (Russell et al. 2011). Thus, the presence of active Tse1 and Tse3 proteins had an ecological advantage in competition studies with *P. putida*. Similar bactericidal functions were assigned to T6SS effectors for have been identified for effector molecules in *Burkholderia thailandensis*, *V. cholerae*, *S. enterica*, and *Bacteroidetes* (Russell et al. 2014).

Expression of T6SS proteins is controlled by quorum sensing and can vary from low cell density to high cell density. Quorum sensing-dependent expression of T6SS was identified in *V. cholerae* (Ishikawa et al. 2009; Zhang et al. 2011), *V. alginolyticus* (Sheng et al. 2012), *V. parahaemolyticus* (Wang et al. 2013a), *P. aeruginosa* (Lesic et al. 2009; Sana et al. 2012), *Yersinia pseudotuberculosis* (Zhang et al. 2011), and *Aeromonas hydrophila* (Khajanchi et al. 2009). In addition to T6SS regulation, quorum sensing systems also regulate starvation adaptation and stress responses (Joelsson et al. 2007).

The master regulator of quorum sensing pathways is a TetR-family transcriptional regulator, HapR (Jobling and Holmes 1997). HapR has been shown to bind to the promoters of the T6SS genes *hcp1* and *hcp2* (Tsou et al. 2009), and deletion of the *hapR* gene strongly repressed Hcp expression. These data support the hypothesis that HapR is a transcriptional activator of Hcp. Deletion of *rpoN* gene in *V. cholerae* strain A1552 also abolished transcription of *hcp1* and *hcp2*, eliminating Hcp expression (Ishikawa et al. 2009). RpoN-binding sites were present in *hcp1* and *hcp2* promoter regions, confirming its role in binding to the *hcp* promoters. In addition, a global regulator, TsrA (VC0070), was found to control T6SS in

V. cholera along with the quorum sensing system (Zheng et al. 2015). It was proposed that the T6SS in *V. anguillarum* served as a sensor for extra-cytoplasmic signals that modulate expression of RpoS, the stress response regulator. *hcp* mutants had lower survival under stress conditions compared to the wild-type strain (Weber et al. 2009).

These studies indicated that quorum sensing, T6SS, and stress/starvation responses are connected by common global regulators. In *P. fluorescens*, Pfo-1 mutation in T6SS-related gene (TssB2 protein COG 3516) affected its ability to survive under arid environment (Varivarn et al. 2013). Similar observations were made on *Azospirillum* defective in polyhydroxyalkanoate production (Dobbelaere et al. 2001). In *P. putida* LS46, Toxin 61 (PPUTLS46_03632), SUKH_6 (PPUTLS46_12325), and Toxin 43 (PPUTLS46_19761) genes were present in the vicinity of T6SS. There is no experimental evidence about the excretion of these toxins via T6SS in *P. putida* LS46. However, in other bacteria, these toxins are secreted by T6SS and have a role in interstrain competition (Jamet and Nassif 2015).

7.9 Polyhydroxyalkanoate Synthesis in *P. putida* LS46

P. putida LS46 was isolated as strain that synthesized mcl-PHAs from a wide range of carbon sources (Sharma et al. 2012; Fu et al. 2014). Mcl-PHAs are accumulated when bacterial growth is limited by deprivation of some essential nutrient such as nitrogen, phosphorous, or oxygen in the presence of a surplus amount of a carbon. PHA polymers are insoluble in water and accumulate as granules inside the cells (Lu et al. 2009; Potter and Steinbüchel 2005; Verlinden et al. 2007).

P. putida strains are soil bacteria that colonize the plant rhizosphere. PHA synthesis appears to be an important trait for root colonization and plant growth promotion in rhizosphere bacteria. *Azospirillum brasilense* inoculants with high PHA contents were more successful in colonization in the plant rhizosphere (Dobbelaere et al. 2001; Helman et al. 2011). Accumulation of PHA in non-Antarctic bacteria helped the bacteria to withstand starvation and survive under hostile environmental conditions (Goh and Tan 2012; Pham et al. 2004).

Bacteria that express T6SS effector proteins have been shown to gain selective advantages within the host and in ecological niches. T6SS effectors proteins provided *P. aeruginosa* with an ecological advantage when competing with other bacteria. The functions described for T6SS in pathogens are overlapping with PHA production in non-pathogens. How these two systems are related is unknown, but some common regulators have been identified.

7.10 Regulation of PHA Synthesis

Regulation of PHA synthesis and accumulation in bacteria is dependent on many physiological and regulatory factors, including transcriptional regulation, redox potential, nutrient limitation, and availability of metabolic intermediates. A TetR-like transcriptional regulator, PhaD, in *P. putida* KT2442 activates the *pha* synthesis cluster genes, *phaC1-Z-C2-D*, also referred to as the *pha* operon (de Eugenio et al. 2010). In *P. oleovorans* GPo1, *phaD* was transcribed as part of the *pha* synthesis operon and was shown to regulate its own transcription, as well as the transcription of the *phaIF* operon, which in turn is involved in the regulation of *pha* synthesis cluster genes (Prieto et al. 1999).

The two-component GacS/GacA system forms the sensory apparatus of the Gac/Rsm cascade, which involves interactions with small RNA regulatory molecules affecting a range of microbial metabolic pathways (Lapouge et al. 2008). GacS/GacA is a sensor kinase that acts in concert with other environmental sensors involved in carbon availability and redox sensing to coordinate the cell's response, for example, by switching the metabolic focus from carbon utilization and growth to carbon storage (Wang et al. 2013a, b).

GacS/GacA-*rsm*, originally detected in *P. putida* CA-3, was also found in the genomes of other *P. putida* strains, with up to 96 % identity with corresponding sequences in *P. putida* KT2440 and *P. putida* F1 (Ryan et al. 2013). Disruption of the *gacS* sensor kinase gene in a *P. putida* CA-3 mutant was found to eliminate mcl-PHA synthesis. Disruption of the *gacA* gene in *P. aeruginosa* resulted in 2- to 17-fold lower levels of transcription of the *pha* operon genes (*phaC1*, *phaZ*, *phaC2*, *phaI*, and *phaF*) compared to wild-type (Pham et al. 2004; Wang et al. 2013b). Genes annotated as components of T6SS were also downregulated (2- to ~100-fold) in the *P. aeruginosa* *gacA* mutant relative to wild-type, suggesting positive control by GacA. *P. aeruginosa* mutants deficient in PHA biosynthesis had similar phenotypes as T6SS defective mutants, such as survival after heat shock (at 50 °C), biofilm formation, and attachment (Pham et al. 2004).

Quorum sensing regulates a number of biosynthetic pathways, including PHA synthesis. Treatment of *P. aeruginosa* cells with the quorum sensing inhibitor, azithromycin, decreased the accumulation of PHAs in *P. aeruginosa* (Xu et al. 2011), and resulted in reduced expression of *gacA* and *rsmA*, which encode the GacA-*rsm* components of the GacS/GacA-*rsm* regulatory cascade (see above), which positively regulates *rsmY* and *rsmZ* which, in turn, regulate quorum sensing (Pérez-Martínez and Haas 2011). Thus, it appears that quorum sensing may regulate PHA synthesis and accumulation by cell-to-cell signal transfer via T6SS.

PHA is accumulated during stationary phase of cell grown under conditions of nutrient (e.g., nitrogen) limitation in the presence of abundant carbon. Intracellular levels of the stress response regulator, RpoS, are very low in the exponential phase and increase in stationary phase (Ryan et al. 2013). RpoS is known to positively regulate PHA synthesis after the onset of the stationary phase or under conditions of nutrient limitation in the presence of abundant carbon. In *P. putida*, KT2440 deletion of the *rpoS* gene was associated with an increase in the expression of

enzymes involved in PHA depolymerization, and the *rpoS* mutant displayed lower survival and tolerance to H₂O₂ stress (Raiger-Lustman and Ruiz 2008). Depolymerized PHAs are catabolized as a source of carbon and energy under nutrient stress conditions. In *P. chlororaphis* PCL1391, mutation of *rpoS* resulted in diminished expression of the *phaC2* gene (Girard et al. 2006). Thus, the stress response regulator, RpoS, appears to play a role in regulation of PHA synthesis. Both RpoS and the quorum sensing regulator, VanT, are been shown to be positively regulated by T6SS proteins (Weber et al. 2008).

Csr, a global carbon storage regulator protein, is involved in the repression of several stationary-phase processes and in the activation of some exponential-phase functions. The CsrA protein regulates about 700 genes including genes for glycolysis/glycogen biosynthesis, fatty acid metabolism, and butanol production in *E. coli* (McKee et al. 2012). Three major components of Csr include an RNA-binding protein CsrA and two small untranslated RNA molecules, *csrB* and *csrC* (Romeo 1998). Both the *csrB* and *csrC* RNAs function as antagonists of *csrA* by sequestering this protein and preventing its binding to mRNA. Overexpression of *csrB* doubled butanol synthesis, while fatty acid production increased by 1.8-fold.

CsrA is also known to regulate the Quorum Sensing regulator Sdi and CstA (carbon starvation regulator protein). The small RNA-binding proteins of the RsmA/CsrA family mediate posttranscriptional regulation of diverse genes required for the starvation response and quorum sensing (Dubey et al. 2003; Morris et al. 2013). The *P. putida* LS46 genome encodes three genes annotated as carbon storage regulators and a protein identified as carbon starvation regulator protein (CstA PUTLS46_012968). Thus, it is highly likely that in *P. putida* strains, including *P. putida* LS46, carbon storage, PHA synthesis, and accumulation, are regulated by the T6SS and by quorum sensing via CsrA. Thus, these data suggest a new role for T6SS proteins in the ecology of bacteria and suggest that signal-sensing mechanisms (quorum sensing and T6SS) modulate the expression of stress response regulators (Weber et al. 2009).

7.11 Crosstalk Communication Among Quorum Sensing, T6SS, and PHA Synthesis

The role of T6SS effector molecules in virulence has been identified in pathogenic bacteria (Russell et al. 2011, 2014). T6SS systems have also been found to have multiple roles in a wide variety of nonpathogenic bacteria (Jani and Cotter 2010; Lertpiriyapong et al. 2012). *P. putida* is a diverse species of soil bacterium, and different strains of *P. putida* are known to colonize root surface, compete with other bacteria in the rhizosphere, inhibit growth of plant pathogens, and survive under extreme conditions of starvation by accumulating PHAs. Control of some of these functions by T6SS has been demonstrated in some bacteria, but there is no direct evidence to date of the role of T6SS in regulation of metabolic processes in *P. putida*.

Fig. 7.4 Hypothetical model of the role of T6SS in *P. putida* LS46. The *solid* arrow indicates the role, with evidence in *Pseudomonas*, and the *dotted* arrow indicates no evidence for the role



Nonetheless, there is abundant indirect evidence from a variety of studies that suggest T6SS plays a regulatory role in *P. putida*. The convergence of quorum sensing, T6SS, and PHA synthesis suggest their interrelation in different physiological processes, including PHA accumulation. On the basis of these observations, we have proposed a hypothetical model to show how T6SS effectors can communicate with other cellular functions. Earlier studies have confirmed the role of RpoS, a stationary-phase regulator in PHA synthesis and stress tolerance in *P. chlororaphis*, which also regulates quorum sensing. Studies of *gacA* mutants of *P. putida* CA-3 eliminated PHA synthesis. However, in *gacA* mutants of *P. aeruginosa*, expression of T6SS proteins and PHA synthesis genes were downregulated to the same extent. Is the effect of *gacA* mutations on PHA synthesis direct? or mediated by T6SS signaling? Furthermore, CsrA, the carbon storage regulator protein, regulates Quorum Sensing and carbon metabolism, including fatty acids in *E. coli*. Keeping these observations in mind, we speculate the GacA/GacS system regulates Quorum Sensing, which in turn regulates T6SS. T6SS effector molecules could be involved in signal transduction within the population for induction of PHA synthesis and accumulation (Fig. 7.4).

7.12 Conclusions

Seven secretion systems (Type I–Type VII) have been reported in Gram-negative bacteria. The Type VI secretion system was first identified in two human pathogens, *P. aeruginosa* PAO1 and *V. cholerae* ATCC 39315. Type VI secretion system components assemble a system for secretion of the hemolysin-coregulated protein (Hcp1) and the valine glycine repeat protein (VgrG). Putative genes encoding T6SS

are present in Gram-negative bacterial genomes belonging to human or plant pathogens, symbiotic nitrogen fixers, and nonpathogenic soil bacteria. *P. putida* LS46 is a nonpathogenic, Gram-negative bacterium, isolated from wastewater that synthesizes mcl-PHAs.

Using the amino acid sequences and COD family identifications of T6SS proteins from *P. aeruginosa* PAO1 as a probe, three putative T6SS operons were identified in *P. putida* LS46. Two of the T6SS gene clusters (S1 and S2) were identical in organization to the T6SS genes of *P. aeruginosa* PAO1, and their gene products showed a high degree of amino acid sequence identity. The third T6SS gene cluster (S3) had a different organization. Some genes of the T6SS S1 and S2 operons were not present in the S3 operon. The organization of the T6SS S1, S2, and S3 operons was highly conserved among all *P. putida* strains examined, except *P. putida* BIRD1. Moreover, the amino acid sequences of the gene products encoded in the T6SS S1, S2, and S3 operons of these *P. putida* strains was also highly conserved. In contrast, amino acid sequences of the gene products encoded by the *P. putida* LS46 T6SS S1, S2, and S3 operons had low identities with the amino acid sequences of T6SS gene products encoded by *Vibrio cholerae*, *Aeromonas hydrophila*, *Rhizobium leguminosarum*, and *Burkholderia mallei*.

Thus, the T6SS genes of *P. putida* and other soil bacteria may have functions that are different from pathogenic *Pseudomonas* species. Recent findings have indicated a role for T6SS in cell-to-cell communication (quorum sensing) beyond regulation of interactions between the bacteria and their eukaryotic hosts. Three carbon storage regulators (RpoS, CsrA, and quorum sensing proteins) are present in the *P. putida* LS46 genome, and we suggest that T6SS proteins regulate the activity of genes associated with carbon metabolism and PHA synthesis.

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Rachhpal S. Kahlon

Contents

8.1	Introduction	282
8.2	Basic Physiology and Metabolism of <i>Pseudomonas</i>	283
8.3	Current Biology of <i>Pseudomonas</i>	285
8.3.1	Genetic Engineering and Systems Biology	287
8.4	Industrial Applications	291
8.4.1	Fine Chemicals	291
8.4.2	Bioactive Compounds	295
8.4.3	Enzymes and Biocatalysis	298
8.5	Biopolymers	303
8.5.1	Bioplastics: Polyhydroxyalkanoates	303
8.5.2	Alginate	311
8.6	Biosurfactants	313
8.6.1	Rhamnolipids	314
8.6.2	Applications of Biosurfactants	319
8.7	Natural Products by Heterologous Gene Expression	321
8.8	Environmental Applications of <i>Pseudomonas</i>	323
8.9	Agricultural Applications	324
8.9.1	Biocontrol of Phytopathogens	324
8.9.2	Insecticidal Activity of <i>Pseudomonas fluorescens</i>	326
8.10	Conclusion	326
	References	327

Abstract

Pseudomonas are known for their ubiquitous nature and metabolic diversity enabling them to survive in a wide range of ecological niches in terrestrial and marine environments as well as in association with animals and plants. They are

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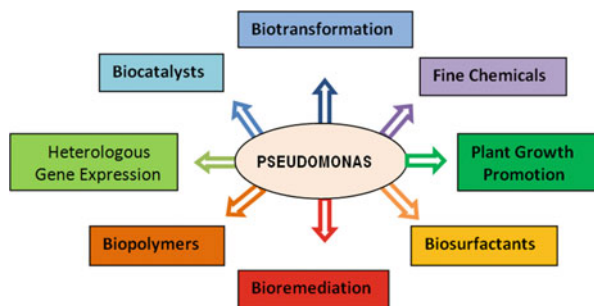
characterized with enormous biosynthetic capacity and production of a wide range of secondary metabolites and are endowed with an array of enzymes and metabolic pathways. Genomes of about 70 different strains of *Pseudomonas* spp. have been fully sequenced, and their vast genetic potential for biotechnological applications such as synthesis of small molecular weight chiral compounds, biopolymer production, bioremediation of organic chemical pollutants, biocontrol of plant diseases and soilborne pathogens and lately for production of heterologous proteins and molecules has been elucidated. *Pseudomonas* also show high degree of robustness and tolerance to extreme environmental conditions of temperature, pH, presence of toxins and solvents required for industrial exploitation. *Pseudomonas putida* KT2440 has been certified as ‘generally regarded as safe’ (GRAS) for gene cloning and expression of foreign DNA, thus providing novel possibilities to engineer this organism not only for industrial applications but also for production of heterologous proteins. Techniques of system biology and expanding genomic databases will go a long way to expand the range of products formed by manipulating the metabolic pathways and develop as efficient cell factories.

8.1 Introduction

Genus *Pseudomonas* represents a metabolically versatile and diverse group of gamma-proteobacteria which occupy a variety of niches in nature and are important from point of view of medical sciences, environment, agriculture and industrial biotechnology. Their metabolic capacity and genetic diversity are reflected by their ability to (1) adapt to diverse and challenging environment, (2) degrade a variety of recalcitrant and toxic chemicals and (3) synthesis of low molecular weight compound as well as biopolymers of industrial importance (Clarke 1982). Besides this, *Pseudomonas putida* and *P. fluorescens* have been found to be suitable hosts for gene cloning and synthesis of heterologous proteins (Poblete-Castro et al. 2012). Presently, the genus *Pseudomonas* (*sensu stricto*) comprises of over 200 species, and many species earlier classified as *Pseudomonas* (Stanier et al. 1966; Palleroni 1984) have been assigned to existing or new genera under other subclasses of class *Proteobacteria* (Kerster et al. 1996; Anzai et al. 2000). For details, reader is referred to Chap. 1.

The ubiquitous nature of this vast group coupled with broad biocatalytic potential and genetic diversity makes *Pseudomonas* a highly valuable bio-resource for exploitation of their metabolic potential for biotransformations, synthesis of high-value chiral compounds for biosynthesis, production of biopolymers and biosurfactant agents, degradation of persistent organic pollutants and a wide range of biotechnological applications in environmental bioremediation and agriculture as biocontrol agents. Particularly the strains with a feasibility to tolerate various types of biological and abiological stresses are important. *Pseudomonas*

Fig. 8.1 Important areas of application of *Pseudomonas*



putida S12 strain has evolved several mechanisms to tolerate solvents such as toluene and deal with toxic solutes by modification of the inner and outer membranes and extrusion of a broad range of aromatic compounds. The membrane-bound efflux pump systems have been detected in several pseudomonads. Such organisms offer a wider degree of freedom and can be successfully used in the production of toxic-substituted aromatic compounds from toxic compounds such as toluene (de Bont 1998; Rojas et al. 2001, 2004). Potential areas of application of *Pseudomonas* are presented in Fig. 8.1.

8.2 Basic Physiology and Metabolism of *Pseudomonas*

The variety of niches in the natural environment inhabited by *Pseudomonas* spp. reflects the diversity of their metabolism and ability to adapt to physicochemical and nutritional situations. Strains of *P. putida* have the capacity to utilize more than 80 organic substrates as sources of carbon and energy, while some other species can utilize even a higher number. Glucose is metabolized by Entner-Doudoroff pathway but is not a preferred substrate as compared to organic acids. The Embden-Meyerhof-Parnas (EMP) pathway for carbohydrate utilization is not operative as *Pseudomonas* lack the key enzyme, 6-phosphofructokinase (*pfk*) and a number of regulatory mechanisms such as HexR of the EMP pathway (del Castillo et al. 2008). The metabolism of glucose which is transported by ABC (ATP-binding cassette) uptake system begins as it enters the periplasmic space where it is oxidized to gluconate and 2-ketogluconate by glucose dehydrogenase (*gcd*). All the three routes of glucose metabolism operate simultaneously, and the three compounds are transported through the cytoplasmic membrane by ABC uptake system and phosphorylated to glucose-6-phosphate, gluconate-6-phosphate and 2-ketogluconate-6-phosphate by specific kinases (del Castillo et al. 2007). Gluconate-6-phosphate is the key intermediate of the ED pathway as well as it feeds the pentose phosphate pathway for biosynthesis. Glucose-6-phosphate and 2-ketogluconate-6-phosphate also converge to gluconate-6-phosphate (Fig. 8.2).

The ED pathway yields two 3C compounds, the glyceraldehyde-3-phosphate and pyruvate per molecule of glucose. The pentose phosphate pathway primarily

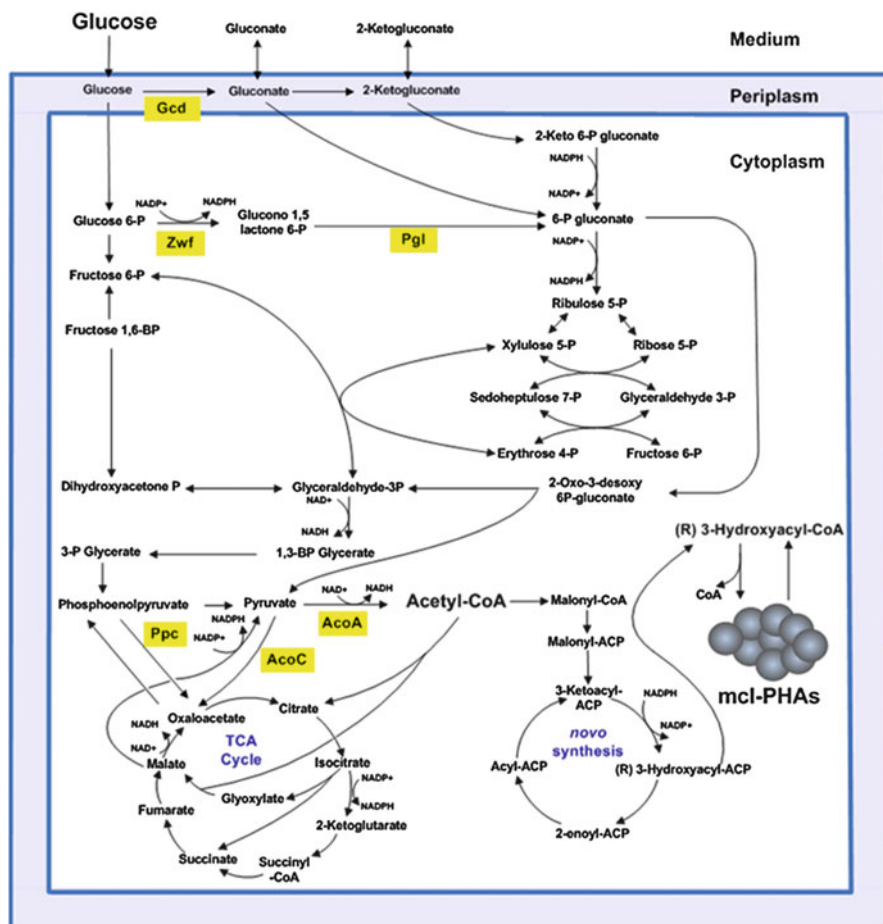


Fig. 8.2 Central metabolic pathways of *P. putida* KT2440 and synthesis of mcl-PHA

provides precursors for biosynthesis. The pyruvate either yields acetyl-CoA or uses pyruvate shunt pathway to feed oxaloacetate rather than the direct oxidation of malate by malate dehydrogenase and enters the TCA cycle. Besides sugars, *Pseudomonas* also metabolizes a number of other substrates such as fatty acids, polyols (such as glycerol), amino acids and a range of aromatic compounds which all enter the central metabolic routes. Glyoxylate shunt of TCA cycle is important in anaplerotic reactions (Ebert et al. 2011). Glucose metabolism by ED pathway is efficient in the supply of reducing power which is an important feature for industrial biocatalysis using *Pseudomonas*. The increased levels of intracellular NADH result in higher production of alkanoates. An increase in $\text{NADH}/\text{NAD}^+\text{P}$ ratio favours anabolic reactions. Furthermore, *Pseudomonas* have the capacity to utilize variety of natural and unnatural aromatic compounds generally referred as peripheral pathways which converge at catechol/protocatechuate/gentisate/homogentisate/

hydroquinone which are further metabolized and enter the central metabolic pathways. A number of plasmids have been identified to mediate the metabolism of aromatic compounds and also provide a means of fast spread among populations by horizontal gene transfer (HRT) (Jimenez et al. 2002). The central carbon metabolism and balance of reducing power and energy metabolism are important aspects of synthesis of industrially important product. Consequently, the solvent-tolerant *P. putida* DOT-TIE responds to drastically increased demand for energy in the presence of toxic solvent by enhanced generation of NAD(P)H coupled with increase in glucose uptake and reduced anabolic demand, a major feature for industrial application of *Pseudomonas* (Wierckx et al. 2005).

Development of the genome sequencing methods and technology provided an efficient and robust system to understand the underlying metabolic and genetic systems, and with this genus *Pseudomonas* has emerged as an important group in biotechnology. *Pseudomonas aeruginosa* PA01 was the 25th bacterium whose complete sequence was determined (Stover et al. 2000). Today, complete genome sequences of about 70 strains spanning different species are available on different data banks (www.pseudomonas.com). *Pseudomonas putida* is a non-pathogenic versatile bacterium that colonizes many different environments and is known for its vast metabolic potential and genetic plasticity (Clarke 1982). *Pseudomonas putida* KT2440, a TOL minus derivative of *P. putida* mt-2 (Franklin et al. 1981), is recognized as an efficient host-vector biosafety system for cloning and expression of heterologous genes (Ramos et al. 1987; Cases and de Lorenzo 1998). This strain has been extensively used for exploiting the potential biotechnological applications such as bioremediation of environment contaminated with toxic recalcitrant compounds (Timmis et al. 1994; Nikel and de Lorenzo 2013), biomining and desulphurisation of fossil fuels (Galán et al. 2000; Brune and Bayer 2012), biocatalytic production of fine chemicals (Schmid et al. 2001; Ouyang et al. 2007a, b), production of bioplastics (Olivera et al. 2001) and as agents for plant growth promotion and biocontrol (O'Sullivan and O'Gara 1992; Walsh et al. 2001; Loper et al. 2012).

8.3 Current Biology of *Pseudomonas*

Genomic sequences complemented with data on proteomics, transcriptomics and fluxomics have led to in silico analysis and modelling on organism with different biotechnological capabilities (Nelson et al. 2002; Jimenez et al. 2002; Yuste et al. 2006). Metabolic reconstruction is a unique tool to target the organism, and many different condition-specific models can be derived within the specific physicochemical and environmental boundaries (Price et al. 2004; Reed et al. 2006; Becker et al. 2007). Genome-scale metabolic reconstruction of *P. putida* KT2440 was undertaken on the basis of genomic, biochemical and physiological information (Nogales et al. 2008). The reconstruction of *P. putida* KT2440 into *iJN746* (*P. putida* KT2440:*iJN746*) as a cell factory included 746 genes, 950 reactions and 911 metabolites spanning over 55 specific pathways or subsystems. Based on their

functional role, *iJN746* captured all the biotechnologically relevant pathways pertaining to polyhydroxyalkanoate synthesis and catabolic pathways of aromatic compounds (e.g. toluene, benzoate, phenyl acetate, nicotinate). In general, the transport subsystem had the highest number of gene-associated reactions which conforms to 12 % of the *P. putida* genome to encode transport-associated gene products. Reactions related to amino acid metabolism were also found to be important as de novo synthesis pathways for all the amino acids are present in *P. putida*, besides many amino acids are also utilized as sources of carbon and energy. Catabolic pathways of aromatic compounds reflect another important physiological ability as these are used as carbon and energy sources. Despite the loss of TOL plasmid and consequent TOL pathway in KT2440, the plasmid genes were included in the *P. putida* metabolic reconstruction since the TOL plasmid is present in the parent strain *P. putida* mt-2 and extensively used to expand the metabolic potential of *P. putida* KT2440. Another subsystem group is the lipid metabolism which is involved in the biosynthesis and accumulation of medium-side-chain polyhydroxyalkanoates (mcsPHAs), the precursors for bioplastics. The two subsystems of lipid metabolism and mcsPHA accumulation and aromatic catabolic pathways are important biotechnologically (Madison and Huisman 1999; Hazer and Steinbüchel 2007; Oberhardt et al. 2008). The reconstructed *iJN746* strain of *P. putida* is comparable with other network reconstructions such as *E. coli*, iAF1260; *B. subtilis*, iVo844; *M. tuberculosis*, iNJ661; and *P. aeruginosa*, iM1056 (Nogales et al. 2008). *Pseudomonas putida* and *P. aeruginosa* show significant differences in their lifestyle and metabolic capabilities. *P. aeruginosa* being a pathogen contains a pathway necessary for growth and virulence factors including alginate, rhamnolipids, phenazine and quorum sensing which are absent in *P. putida* reconstruct. In contrast to this *P. putida* has genes necessary for degradation of aromatic compounds which are absent in *P. aeruginosa*. Consequently, a larger number of genes were included in the metabolic constructs, i.e. 14 % of the genome of *P. putida* compared to 18 % of the genome of *P. aeruginosa* (Nogales et al. 2008). Metabolic versatility of *iJN746* was assessed by Flux Balance Analysis (FBA) and in silico comparison of growth performance. The metabolic versatility is reflected by growth stimulation by 59 different carbon sources in IM9 minimal medium and metabolism of aromatic compounds in the construct has biotechnological significance. *Pseudomonas putida* also shows preference for rhizosphere colonization because of enhanced and ready availability of amino acids, organic acids and aromatic compounds derived from seed or roots of the plants.

Other biotechnologically important process is the capacity of the metabolic network to form poly-3-hydroxyalkanoates (PHA) that can be used for production of bioplastics as a biodegradable substitute to plastics derived from petrochemicals. The medium-side-chain PHA (mcsPHA) ranging between C₆ and C₁₆ 3-hydroxy fatty acids are commonly produced by fluorescent pseudomonads, and *P. putida* KT2440 is an excellent candidate for mcsPHA production. *iJN746* accounts for production of mcsPHAs in the range of C₆ to C₁₄ from a variety of substrates used as carbon and energy sources. The carbon sources yielding acetyl-CoA are a good

source of PHA production. These include (1) L-branched-chain amino acids (L-leucine, L-isoleucine, L-valine, etc.) and (2) aromatic compounds metabolized via β -ketoadipate pathway (catechol, *p*-coumarate, etc.); besides, these phenyl acetic acid and glycerol are excellent precursors for PHA (Ward et al. 2005). Overall fatty acids give the highest yield of PHA per carbon unit of the substrate.

8.3.1 Genetic Engineering and Systems Biology

With the identification and genetic characterization of degradative plasmids in *Pseudomonas*, a lot of attention was diverted towards metabolism of xenobiotic compounds by *Pseudomonas* and related bacteria. Degradation of halogenated aromatics, organic solvents and polycyclic and heterocyclic compounds was particularly important. These were extensively used as insecticides in agriculture and solvents in industry and in biosynthesis. A number of strains have also been identified to degrade pesticides such as 2,4-D, 2,4,5-T, γ -BHC, atrazine and co-metabolize different fractions of DDT. The metabolic capacity of many strains was extended by pooling plasmids or genetic engineering of strains for modification of enzyme specificity and affinity, pathway construction, regulation and bioprocess development for their potential for bioremediation (Pieper and Reineke 2000).

First hybrid pathway was constructed in *Pseudomonas* sp. B13 (*P. knackmussii* B13) having plasmid PWR1 encoding 3-chlorobenzoate and 4-chlorophenol metabolism, by conjugal transfer of plasmid PWWO from *P. putida* mt-2. The substrate potential of B13 was also extended by cloning *xylXYZ* (toluate 1,2-dioxygenase) and *xylL* (carboxylate dehydrogenase) genes from TOL plasmid (PWWO-161) of *P. putida* and *nahG* gene (salicylate-hydroxylase) from NAH7 plasmid (Worsey and Williams 1975; Lehrbach et al. 1984). Hybrid plasmid with genes *xylXYZL*, regulatory gene *xylS* and the promoter pM was introduced into B13 by conjugation. The exconjugant *Pseudomonas* sp. B13 (TOL) was able to utilize 3-chloro-, 4-chloro-, 3,5-dichlorobenzoic acid. The trans-conjugants containing *nahG* gene were able to degrade salicylate and its chlorinated derivatives, the 3-, 4- and 5-chloro-salicylate. Similarly, introduction of dehalogenase genes into the biphenyl degrading strain, *Comamonas testosteroni* VP44, resulted in complete mineralization of ortho- and para-substituted monochlorobiphenyls (Harayama et al. 1999). Field application vectors (FAV) are a combination of a selective substrate that can be used easily by a host microorganism and a cloning vector to provide a temporary niche for the host organism in harsh environment. FAV can stabilize and enhance the expression of foreign genes in contaminated sites. The chromosomally borne gene *bphABC* encoding PCB catabolism in *Pseudomonas* sp. strain ENV307 was cloned into broad host range plasmid pRK293. This plasmid was transferred into *Sphingomonas paucimobilis* IIGP capable of degrading nonionic surfactant Igsal CO-720[®] (IGP) as a selective substrate. The recombinant strain IIGP4 (PCL3) exhibited the ability to degrade PCB congeners in Aroclor 1242[®] without biphenyl as an inducer. The transposon-encoded PCB catabolic genes (*pbhABC*) were more

stable than plasmid-encoded genes after insertion into the surfactant-utilizing strain, *P. putida* 1PL5 (Lajoie et al. 1994).

Thus using genetic manipulation, construction of strains for bioremediation of biphenyls, benzene, phenols and their chlorinated derivatives and nitroaromatics have been reported (Menn et al. 2010). However, such cultures do well in isolation but may fail in competition with other microorganisms in the natural environment where they exist as communities of multiple species that are capable of performing more complex tasks than clonal populations as different organisms in the population may complement the metabolic reactions and pathways. The natural consortia play a crucial role as indicated by the human gut microbiome and marine microbe community analysis (Kau et al. 2011; Giovannoni and Vergin 2012). Natural consortia have been selected for better performance and desired properties in areas of dairy processing, fermentations, biogas production and biomining (Lynd et al. 2002; Di Maio et al. 2012; Reid 2012).

Using synthetic biology tools, construction and analysis of synthetic gene circuits have resulted in better understanding of the naturally occurring circuits, their evolution and properties. Thus engineering of synthetic consortia is expected to allow more complex tasks in industrial applications (Momeni et al. 2011; Brenner et al. 2008; Sabra et al. 2010; Shong et al. 2012). Areas such as biomining and bioremediation of heavy metal contamination could benefit immensely from synthetic consortia to enhance mineral recovery. Thus the design and construction of synthetic and mixed microbial consortia will emerge as a powerful tool in optimizing industrial processes as well as provide an insight into the evolution and emergence of naturally occurring microbial consortia. Engineered consortia have great potential in degradation of recalcitrant compounds and bioremediation in the natural environment. Heterologous gene expression is another important aspect of modern biology and metabolic engineering in which we assume that the DNA acts as a sort of software which is read by the reading machine (the host) when entered. Thus the gene expression will take place at the will of the user (Danchin 2012). A number of foreign genes have been expressed in *E. coli* and some other bacteria, and being commercially used, prominent examples are the production of insulin, human growth hormones, interferon, etc. by recombinant strains (Makino et al. 2011).

In view of this concept, the synthetic biology entails the basic, complete genetic and biochemical scaffold needed for gene expression as biologically engineered DNA constructs are plugged in and out for specific purposes. Indeed, the gene expression is subjected to various constraints imposed by the host (1) protein folding sites with certain amino acid sequences fold poorly and saturate the chaperoning ability of the host, (2) the stress caused on endogenous biochemical network by the activities encoded on DNA constructs (e.g. enzymes and their metabolic products) and (3) the drain of metabolic currency that is diverted into production of the metabolic products of the implanted gene(s) and pathways(s), referred as metabolic burden (Silva et al. 2012; Martinez-Garcia et al. 2014).

Thus, the emphasis for improvement of the expression of the genetic implants has been based on refining DNA sequence, i.e. codon usage, promoter strengths,

ribosome binding sites, etc., while the host cell physiology and other biochemical properties have not received much importance. The implanted DNA couldn't function in isolation but has to be integrated into the metabolic machinery of the host as they interact with metabolites and biochemical pathways. The selection of the bacterium as a chassis is analogous to the selection of species for domestication, and innate potential is the most important aspect. Thus, the reliable bacterial chassis for synthetic biology should be derived from bacterial species that naturally evolved with desirable physiological and metabolic properties and are amenable to stable genetic reprogramming. An ideal bacterial chassis has a genome that encodes the basic biological functions required for self-maintenance, growth and stress resistance but is deleted of other cell structures and signal processing components that divert resources. Under natural conditions, these functions enable the organism to interact with their environment and may not be required in biotechnological setting. Thus the strain should be robust and genetically stable (low spontaneous variability) and a strong envelope to endure the harsh conditions of the bioreactor (Foley and Shuler 2010; Danchin 2012).

Although, *E. coli* has been traditionally used for expression of heterologous proteins, the naturally occurring environmental microorganisms, which have evolved through the acquisition and expression of new genes and metabolic pathways for degradation of toxic substances mediated by catabolic plasmids that spread through microbial consortia in polluted sites, appear to be a good alternative. The prominent organism among these is the genus *Pseudomonas* which possesses a vigorous Entner-Doudoroff and pentose phosphate pathways with high NADPH regeneration to counteract the endogenous and exogenous oxygen stresses. This provides a right metabolic framework, and *P. putida* KT2440 is one such strain that is non-pathogenic soil bacterium endowed with remarkable metabolic versatility and tolerance to organic compounds and other stressful conditions such as ROS (reactive oxygen species) (Chavaria et al. 2013).

The high NADPH regeneration rate and other properties enable the evolution and expression of biochemical pathways that would be hardly tolerated by other species of bacteria. The multiple efflux pumps in the cell membrane for efflux of antimicrobial drugs in pathogenic pseudomonads may be used for enhancing solvent tolerance, a property considered important in bioprocessing (Udaondo et al. 2012). *Pseudomonas putida* KT2440 oxidizes organic compounds by peripheral pathways, the products of which enter the central pathways through tricarboxylic acid cycle and glyoxylate cycle. Several of these reactions are coupled to the reduction of NADP^+ to NADPH which are used to generate precursors for biosynthesis of cell material. First metabolic models of KT2440 were given by Nogales et al. (2008) and Puchałka et al. (2008) and have been refined by in silico analysis. The complete metabolic network of KT2440 spans 1070 reactions and 1044 metabolites and is related to 900 genes. A comprehensive interaction database of *P. putida* KT2440 has been generated (Park et al. 2009), which is useful for domestication of this bacterium for industrial exploitation. Such useful traits are encoded by nearly 1500 genes which are common to all *Pseudomonas* species (Loper et al. 2012).

However, this organism suffers from a drawback that of the *innate diversion of the metabolic currency* [ATP and NAD(P)H] into biological functions that are not relevant to industrial processes. Based on these considerations, Martinez-Garcia (2014) developed *P. putida* EM383 strain with desirable properties of biochemical reactions and robustness as a standard chassis for synthetic biology and metabolic engineering for heterologous gene expression as well as holding harsh transformation reactions not feasible with currently used microbial platforms. Nearly 300 genes, representing 4.3 % of genome, were removed from KT2440 to obtain strain EM383. These included energy-consuming whole-flagellar machinery, four prophages, two transposons and three clusters of restriction modification functions spread over 11 chromosomal sites.

Development of strain with reliable and predictable chassis suitable to deliver biotechnological promise of synthetic biology involves many steps such as detection of the traits that are not desirable biotechnologically, enhancement of the desirable traits and incorporation of new activities and regulation. The genetic manipulations will involve deletion/insertions of a single gene to complex genetic constructs and metabolic circuits (Sauer and Mattananovich 2012; Driouch et al. 2012; Nikel et al. 2014).

Pseudomonas putida KT2440 genome comprises of 5420 ORFs, and 76.9 % of this is accredited with functions while the rest represents hypothetical proteins (19.1 %) (Winsor et al. 2011). Thus it requires precise site-specific deletions and replacement with desirable alleles. Martinez-Garcia and colleagues have developed a seamless method for the purpose (Martinez-Garcia and de Lorenzo 2011; 2012). Using this method, genes for synthesis and exporting of flagellar proteins representing 1.1 % of genome (~70 kb) were removed resulting in a nonmotile variant. Up to ~7 % of genome has been erased thus enhancing the adenylate energy charge and NADPH/NADP ratio, a desirable trait for microbial cell factory. For interaction of the desirable characteristics, two plasmid vectors continue to be suitable method for genetic engineering. Plasmid-based vectors have been extensively used in genetic engineering, and a Standard European Vector Architecture (SEVA) has been made available recently as a powerful genetic tool (Silva-Rocha et al. 2013). A strain of *P. putida* has been engineered to degrade pollutant 1,3 dichloroprop-1-ene under limited supply of oxygen. This paved the way for domestication of *P. putida* KT2440 as it is not naturally endowed with the property to thrive in the absence of oxygen as well as degradation of 1,3 dichloroprop-1-ene. Besides plasmids a number of transposon-based vectors are also available which can deliver multiple segments of unlimited size of DNA or one can make use of the integrase activity of the integrative and conjugation elements (ICE) for delivery to specific sites. Stable interaction is an important criterion for the establishment of heterologous genes. Among the environmentally important genomes and metagenomes, a number of transcription factor-promoter pairs are available that respond to chemical factors, e.g. *xylR*, *xylS*, *nahR* and *alkS*, and that respond to xylene, m-toluene, salicylate and short-chain alkanes, respectively (Lee et al. 2005).

Genetic engineering and synthetic biology have come a long way in its endeavour to evolve strains with large-scale biotechnological applications. However,

though the gene structure and function are predictable with reasonable accuracy, there is a lack of understanding of the basic biological facts such as the interplay between genetic programme and the metabolic network. This remains a hurdle for large-scale genetic programming. *Escherichia coli* and *Mycoplasma* spp. have been extensively used as model organisms and are considered most useful models in basic understanding but have limited utility in industrial application because of inherited genome which reflects their limited ecological niches. On the other hand, *P. putida* seems to be superior for generation of designer whole-cell catalysts both for upstream (i.e. biochemical reactions) and downstream (product recovery) processes (Foley and Shuler 2010; Lee et al. 2012a, b).

8.4 Industrial Applications

Members of genus *Pseudomonas* have already been recognized as a potential source for industrial application and production of a number of metabolites, important of which are discussed here to highlight the importance of *Pseudomonas* in industrial biotechnology (Otero and Nielsen 2009; Poblete-Castro et al. 2012; Loeschcke and Thies 2015).

8.4.1 Fine Chemicals

Enzymes are remarkable catalysts which can use an array of complex molecules as substrates and catalyse very specific transformations resulting in myriad of fine chemicals that find applications in food and pharmaceutical industry. Pseudomonads are known for their metabolic versatility and have potential for synthesizing and secreting a variety of small molecular weight compounds using cheap carbon source such as glucose. Oxygenases play an important role in metabolic versatility of *Pseudomonas*. Genomic annotations of *P. putida* KT2440 have shown the presence of 33 putative genes encoding oxygenases (Nelson et al. 2002; Arora et al. 2010). Pseudomonads have been described to uptake, biotransform and catabolize a number of natural products and industrial compounds and funnel the products of these into the central metabolic pathways. Genes and enzymes for the catabolic pathways of catechol, protocatechuate, *p*-hydroxybenzoic acids, etc. have been fully elucidated in a number of strains of *Pseudomonas*. Pathways for the degradation of salicylate, benzoate, camphor, toluene, xylene, naphthalene and their derivatives have reported to be borne on plasmids (Chakrabarty 1976). *Pseudomonas putida* KT2440 is a TOL⁻ derivative of *P. putida* mt2, a TOL⁺ wild type (pWWO) isolated from garden soil has been extensively studied and has emerged as a model organism. Important biotransformation reactions undertaken by *Pseudomonas* spp. are shown in Fig. 8.3.

Pseudomonads have been conceived as cell factories for the production of valuable compounds from cheap substrates as carbon source. *Pseudomonas* can be used for production of low molecular weight chiral compounds with potential

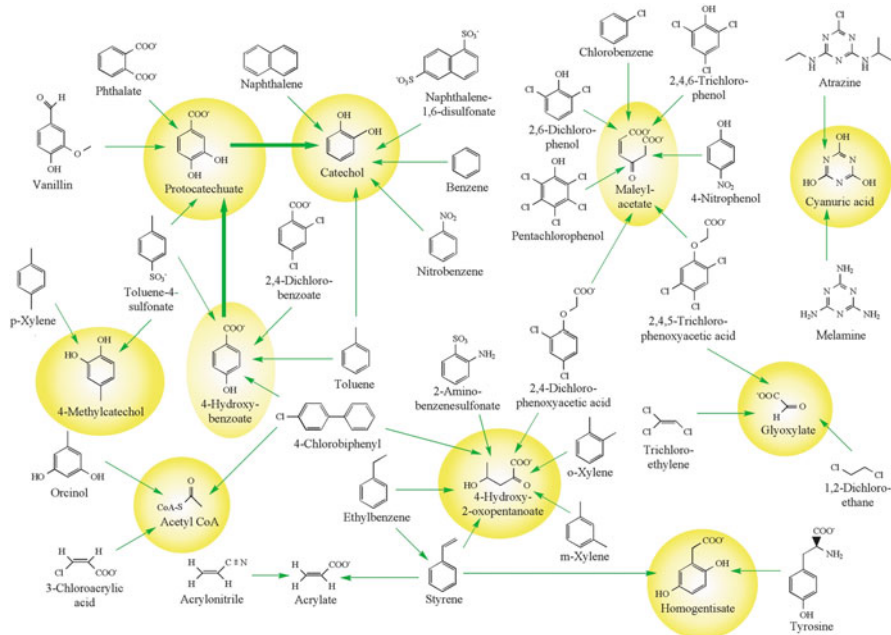


Fig. 8.3 Metabolic network of *Pseudomonas* spp. showing transformation of natural products (vanillin, γ -pinene, limonene, mandelate, camphor and adamantanone) and industrial compounds (bromoxynil, styrene, methyl *tert*-butyl ether (MTBE), trichloroethylene, and nitroglycerin). Metabolites in circles funnel into central metabolic pathways (Wachett 2003 with permission)

use as precursor for the synthesis of pharmaceuticals. To enhance the purity of the compounds to be used as drug, enzymes are used to catalyse the specific reactions. Hydroxylases acting on aromatic compounds are an important group of enzymes used to synthesize chiral compounds. *Pseudomonas putida* KT2440 can metabolize benzoic acid leading to the formation of interesting intermediates of the degradation pathway (Straathof et al. 2002; Jimenez et al. 2002). For example, *cis-cis* muconate, an intermediate of benzoic acid metabolism, is a precursor for adipic acid, used for the synthesis of nylon-6,6 (Draths and Frost 1994; van Duuren et al. 2011, 2012). Toluene dioxygenase (TDO) is an important enzyme catalysing asymmetric *cis*-dihydroxylation of a number of aromatic compounds to *cis*-diols. *Pseudomonas putida* KT2442 (pSPM01) harbouring TDO genes could effectively biotransform a wide range of aromatic substrates into their *cis*-diols products. The oxidation ability of the mutant strain *P. putida* KTOY02 (pSPM01) harbouring TDO gene was increased by cloned expression of *vgb* gene encoding VHb (*Vitreoscilla* haemoglobin) protein to enhance the rate of oxygen uptake (Frey and Kallio 2003). As a result, approximately 42 % more benzene *cis*-diols, 84 % more toluene *cis*-diols and 13 % chlorobenzene *cis*-diols production was achieved in *P. putida* KTOY02 (pSPM01) grown in shake flasks with specific substrates (Ouyang

et al. 2007a, b). Expression of *vgb* has strong effect on cis-diol formation. The diol generated from toluene is used for production of chiral prostaglandin intermediates. Optically active diols can also be generated from quinoline and its derivatives by cis-glycol dehydrogenase-negative mutants of *Pseudomonas* (Davies et al. 1990). Nitrile hydratase identified in *P. chlororaphis* B23 and *Rhodococcus* sp. catalyses conversion of various nitrile compounds to corresponding amines. They are successfully used for industrial production of acrylamide (Meyer et al. 2003).

Monooxygenases are multifunctional enzymes and are involved in reactions such as biodesulfurization, dehalogenation, denitrification and hydroxylation of various aromatic compounds. The few monooxygenases acting on aromatic compounds have been used as biocatalysis for the synthesis of the pharmaceuticals compounds. Examples of these monooxygenases are styrene monooxygenase, hydroxybiphenyl-3-monooxygenase and phenylacetone monooxygenase. Styrene monooxygenase (EC 1.14.13) from *Pseudomonas* sp. VLB 120 has the ability to convert styrene to s-styrene oxide with an enantiomeric purity of 99 % for the synthesis of various chiral acryloxides. *Pseudomonas azelaica* produces a commercially important enzyme, 2-hydroxybiphenyl-3-monooxygenase (HbpA) that catalyses *ortho*-hydroxylation of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl and also catalyses the conversion of different 2-substituted phenols to 3-substituted catechols that are of interest to pharmaceutical industry (Held et al. 1998; Arora et al. 2010). Some important biocatalytic systems used for production of industrial products are listed in Table 8.1.

Pseudomonas putida is commercially used for production of optically active alcohols from achiral or racemic substances, e.g. conversion of isobutyric acid into (s)- β -hydroxybutyric acid. Like wise for chiral 2-aminobutyric acid production, 2-oxobutyric acid is used as the starting material and 2-hydroxy butyric acid is produced from crotonic acid by *P. putida*. L-tartrate, a cheap byproduct of wine

Table 8.1 Biocatalytic systems for the production of industrial products

Organism	Enzyme	Product	Reaction
<i>P. putida</i>	Amidase	Non-proteinogenic amino acid	Kinetic resolution
<i>P. putida</i>	Acylases	Semi-synthetic penicillins	Selective coupling
<i>Pseudomonas</i> sp. DSM8653	Whole cells	4-[6-Hydroxypyridine-3-yl]-4-oxobutyrate	Complex reaction
<i>P. putida</i> ATCC33015	Whole cells	5-Methyl pyrazine-2-carboxylic acid	Xylene oxidation pathway
<i>P. putida</i> KT2440	Engineered strains	Chiral <i>cis</i> -diols	Dioxygenase
<i>P. denitrificans</i>	Whole cells	3-hydroxy propionic acid	Hydratase and dehydrogenase
<i>P. putida</i> S12 (solvent tolerant)	Whole cells	Phenol and cinnamic acid	Enhanced flux towards tyrosine
<i>P. putida</i>	Whole cells	<i>p</i> -hydroxybenzoic acid	Oxidation

Schmid et al. (2001)

industry, can be transformed into D-glyceric acid used for enzymatic production of serine and chemical synthesis of organic compounds (Furuyoshi et al. 1991; Gross et al. 2010).

Genomic analysis of *P. putida* has provided further insight into genetics and metabolism of *Pseudomonas* sp. Bioinformatics has proved useful in predicting the metabolic sequences and in silico modelling for the development of suitable biocatalytic system for the synthesis of organic chemicals. Such processes will prove a boon for biotechnology (Poblete-Castro et al. 2013).

Strains of *P. putida* tolerant to organic solvents have been developed for successful epoxidation of styrene by *P. putida* strain DOT-TIE (Blank et al. 2008), O-cresol production from toluene with *P. putida* T-57 (Faizal et al. 2005) and de novo synthesis of *p*-coumarate (Nijkamp et al. 2007), *p*-hydroxybenzoic acid (Verhoef et al. 2007) or *p*-hydroxystyrene (Verhoef et al. 2009) using *P. putida* strain S12.

Metabolic engineering techniques were used for introduction of L-phenylalanine/L-tyrosine ammonia lyase (*pal*) gene and *p*-coumaric acid decarboxylase (*pdc*) gene into *p*-coumarate producing strain S-12 for *p*-hydroxystyrene production, and the *fcs* gene encoding feruloyl-coenzyme A synthetase was inactivated to prevent degradation of *p*-coumarate intermediate. Two-phase liquid-liquid cultivation system comprising of an aqueous phase and solvent phase along with reactor configuration has been very successful to enhance the productivity (Schmid et al. 2001; Verhoef et al. 2007; Ouyang et al. 2007a, b).

Pseudomonas denitrificans when grown aerobically, i.e. the conditions suitable for regeneration of NAD(P)^+ , produces 3-hydroxypropionic acid (3HP) from glycerol through the two sequential enzymatic reactions catalysed by a coenzyme B_{12} -dependent glycerol dehydratase and NAD(P)^+ -dependent aldehyde dehydrogenase (ALD). A recombinant strain *P. denitrificans* was constructed by cloning genes for glycerol dehydratase (DhaB), and glycerol dehydratase reactivase (GdrAB) from *Klebsiella pneumonia* produced 37.3 mmol/L with 62 % (mol/mol) yield on glycerol. Overexpression of ALDH was not required; however, heterologous expression of ALDH gene (*puu C*) from *K. pneumoniae* in *P. denitrificans* resulted in further improvement in titre and yield of 3HP to 54.7 mmol/L and 67 % (mol/mol) (Zhou et al. 2013).

Bioproduction of Phenol Phenol is another industrially important hydroxylated aromatic compounds as it is used for production of bisphenol A and phenolic resins. Annually over seven million tons of phenol is produced by chemical oxidation of cumene which is produced from benzene. This is an energy-intensive process coupled with hazardous waste. The solvent-tolerant *P. putida* S12 has been identified for conversion of tyrosine into phenol by pyridoxal 5'-phosphate-dependent enzyme tyrosine phenol lyase (TPL). The gene *tpl* identified in *Pantoea agglomerans* was introduced into *P. putida*. Tyrosine phenol lyase (TPL) results in formation of phenol, pyruvate and ammonia from tyrosine. Further, targeted by genetic manipulation, random mutation, antimetabolite selection and high-throughput screening resulted in selection of strain S12TPL3 which can utilize

Table 8.2 Industrial processes based on *Pseudomonas* strains

Biocatalyst	Product	Utility	Company	Source
<i>P. putida</i> ATCC33015	2-Quinoxaline-carboxylic acid	Biological activity	Pfizer	Wong et al. (2002)
<i>Pseudomonas</i> DMS8246	Chiral amines	Biological activity	BASF (Germany)	Schulze and Wubbolts (1999)
<i>P. putida</i>	5-Cyanopentanamide	Catalysis	DuPont USA	Sueglitz et al. (1996)
<i>P. putida</i> ATCC33015	5-Methylpyrazine-2-carboxylic acid	Pharmaceutical	Lonza (Switzerland)	Kiener (1992)
<i>P. putida</i> ATCC12633	Chiral compounds	Pharmaceutical	DSM (Netherlands)	Hermes et al. (1993)
<i>Pseudomonas</i> lipase AK	Paclitaxel	Pharmaceutical	Bristol Myers Squibb	Patel et al. (1994)
<i>P. putida</i>	D- <i>p</i> -Hydroxyphenyl-glycine	Pharmaceutical	Many	Schulze and Wubbolts (1999)
<i>P. putida</i> DSM8653	4-[6-Hydroxypyridine-3-yl]-4-oxobutyrate	Pharmaceutical	Lonza (Switzerland)	Schmid et al. (2001)
<i>Pseudomonas</i>	[5]-2-Chloropropionic acid	Herbicide	Astra. Zeneca (USA)	Schulze and Wubbolts (1999)

glucose, a cheap feedstock for phenol production with a yield of 7 % (mol/mol) in the biphasic growth medium (Wierckx et al. 2005, 2008). This has paved the way for ‘green’ production of hydroxylated, toxic aromatic compounds. Solvent-tolerant bacteria have been used to produce aromatic compounds such as cinnamic acid, *p*-hydroxybenzoic acid and methylcatechol (Wery et al. 2000; Ramos-Gonzalez et al. 2003; Reva et al. 2006; Nijkamp et al. 2005). Important chemicals utilized for synthesis of pharmaceuticals and allied compounds produced commercially by using *Pseudomonas* spp. are listed in Table 8.2.

8.4.2 Bioactive Compounds

Members of genus *Pseudomonas* produce about 800 bioactive substances which include 600 antibiotics and the rest with bioactive properties other than antibiotics (Berdy 2005). A variety of bioactive substances produced by *Pseudomonas* are pyrroles, pseudopeptide, pyrrolidinedione, phloroglucinol, phenazine, benzaldehyde, quinoline, quinolone, phenanthrene, phthalate, andrimid, moiramides, zafrin and bushrin (Romanenko et al. 2008). Some of these show antimicrobial activity, while dibutyl phthalate and di(2-ethylhexyl) phthalate are cathepsin B inhibitors. Cathepsin B is cystine proteinase which plays an important role in pathologies

including inflammation, pancreatitis, tumour angiogenesis and neuronal diseases. The antibacterial metabolites of *Pseudomonas* show diverse mechanisms of action as they affect cell membrane resulting in cell lysis or inhibit enzymes such as acetyl-coA carboxylase and nitrous oxide synthesis.

Pseudomonas bromoutilis produces a pyrrole antibiotic, 2,3,4-tribromo-5 (1-hydroxy,1',4'dibromophenyl) pyrrole, an inhibitor of gram-positive bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae* and *S. pyogenes* at 0.0063 µg/ml and *Mycobacterium tuberculosis* at 0.2 µg/ml (Burkholder et al. 1966). *Pseudomonas synxantha* produces a long-chain hydrocarbon with terminal alyl bond and an intermediate electronegative atom with strong bioactivity. The compound exhibited strong growth inhibitory activities against *M. smegmatis* and *Mycobacterium tuberculosis* strains H37Ra, H37Rv and BCG. Cells of *P. synxantha* and its metabolites show haemolytic activity on human blood. This indicates that the bioactive compound produced by *P. synxantha* has biosurfactant activities as well as anti-mycobacterial properties (Mukherjee et al. 2012, 2014).

Pseudomonas sp. 102–3 isolated from seawater produces, 4-hydroxy-benzaldehyde, 2-*n*-heptyl-4-quinolinol and 2-*n*-pentyl-4-quinol. The 4-hydroxy-benzaldehyde shows low antimicrobial activity and inhibits *Candida albicans*, *S. aureus* and *Vibrio harveyi* at 5 mg/disc, while the other two compounds show a strong antimicrobial activity against *S. aureus*, *V. harveyi* and *V. anguillarum* at 50 µg/disc. Terrestrial *P. aeruginosa* is also known to produce antibiotic 2-*n*-pentyl-4-quinolinol, thus the two organisms have similar biosynthetic capabilities (Wratten et al. 1977).

Pseudomonas fluorescens isolated from marine waters produce moiramides A, B and C besides the andrimid, a pseudopeptide pyrrolidinedione antibiotic (Needham et al. 1994). Moiramide B and andrimid show broad spectrum antibacterial activity against *S. aureus*, *S. pneumoniae* and *E. coli* by inhibition of enzyme acetyl-CoA carboxylase (Freiberg et al. 2006). These compounds show potential as new class of antibiotics to treat bacterial infections.

Pseudomonas sp. 1531-E7 isolated from sponge, *Homophymia* sp., produces quinolones (2-undecyl-4-quinolone; 2 undecen-18-yl-4-quinolone, 2-nonyl-4-quinolone and 2-nonyl-4-hydroxyquinoline *N*-oxide) (Bultel-Poncé et al. 1999). 2-Undecyl-4-quinolone, 2-undecen-18-yl-4-quinolone and 2-nonyl-4-quinolone show anti-amoebic activity against *Plasmodium falciparum*. 2-Undecen-18-yl-4-quinone and 2-nonyl-4-hydroxyquinoline *N*-oxide show toxicity against KB cells. Furthermore, 2-undecyl-4-quinolone and 2-nonyl-4-hydroxyquinoline *N*-oxide are active against HIV-1 and *S. aureus*.

Pseudomonas sp. AMSN produces 2,4-diacetylphloroglucinol (DAPG), a potent antibiotic against methicillin-resistant *S. aureus* (MRSA) with MIC range of 0.25–1.0 µg ml⁻¹. Its bactericidal action is comparable to that of vancomycin. DAPG has been reported to be active against vancomycin-resistant strains of *S. aureus* (VRSA) and *Enterococcus* spp. (VRE) genotype A and B (Isnansetyo et al. 2003; Isnansetyo and Kamei 2009). *P. aeruginosa* isolated from Antarctic sponge produces six diketopiperazines and two phenazine alkaloids. Phenazines are pigments with antibiotic properties. These phenazine alkaloids are active against

B. cereus, *Micrococcus luteus* and *S. aureus*. The diketopiperazines show cytotoxic properties and don't exhibit antibiotic properties. *P. aeruginosa* also produce pigments pyocyanin and pyorubin which exhibit antibacterial activity against *Citrobacter* sp. (Leisinger and Margaraff 1979).

Pseudomonas sp. WAK-1 produces a sulphated polysaccharide (B-1) having repeating units: $-(2)\text{-}\beta\text{-D-Galp(4SO}_4\text{)(1-4)[}\beta\text{-D-Glcp(1-6)]-}\beta\text{-D-Galp(3SO}_4\text{)(1-}$, which is active against herpes simplex virus 1 (HSV-1) and also shows anticancer properties on cell lines of central nervous system and lung cancer (Matsuda et al. 2003).

Pseudomonas stutzeri CMG1030 produces two novel substances, zafrin [4b-methyl-5, 6, 7, 8 tetrahydro-1 (4b-H)-phenanthrenone] and bushrin (7-(3-furyl)-3,7-dimethyl-7,8-dihydro-1-naphthalenol) with antimicrobial activity. Zafrin is active against *Salmonella typhi* and *S. aureus* but not against *Candida albicans*. In contrast to this, bushrin is more versatile and is active against a number of gram-positive and gram-negative bacteria, viz. *B. subtilis*, *B. stearothermophilus*, *B. cereus*, *Clostridium perfringens*, *C. sporogenes*, *S. aureus* (MSSA and MRSA), *S. epidermidis*, *E. faecalis*, *E. faecium*, *V. harveyi*, *S. typhi*, *P. mirabilis*, *P. morgani*, *Shewanella putrefaciens*, *Hafnia alvei* and *Serratia marcescens*, however, inactive against *C. albicans*, *Klebsiella aerogenes* and *Streptococcus* group G. Antibacterial activity of this substance results in cell lysis (Uzair et al. 2006, 2008).

Pseudomonas strain I-2, isolated from marine environment produces a nonprotein chloroform-soluble substance active against vibriosis of shrimps caused by *V. harveyi*, *V. fluvialis*, *V. parahaemolyticus*, *V. damsela* and *V. vulnificus*. *Pseudomonas* sp. I-2 is non-pathogenic to shrimp larvae and may be useful as biocontrol agent against vibriosis of shrimps (Chythanya et al. 2002).

Recently, *P. putida* isolated from rhizosphere soil was reported to produce an antibiotic and antitumor phenazine derivative, 5-methyl-phenazine-1-carboxylic acid (Kennedy et al. 2015). *Pseudomonas* sp. PB2 exhibits anti-angiogenic haemolytic, cytotoxic and antimicrobial activities against *S. aureus*, *S. epidermidis*, *S. luteus*, *E. coli* and *Candida albicans* and exhibit cytotoxic activity on PC12 and HeLa cells (Thakur et al. 2005). *Pseudomonas fluorescens* HAK-13 produces active proteinaceous substances having lytic activity against algae and has potential as a biocontrol agent for harmful algae blooms in marine environment (Kim et al. 2007b).

Three bioactive compounds, 1-hydroxyphenazine, phenazine-1-carboxylic acid (PCA) and rhamnolipid-I (RL-1), have been identified from *Pseudomonas* sp. strain ICTB-745 isolated from Ladakh, India, having extreme cold desert-like conditions at an altitude of 12,000 m from sea level (Ahmed et al. 2012). These compounds exhibited antimicrobial as well as cytotoxic activities and biopesticide properties. Though all the four metabolites showed antibacterial activity against *S. aureus*, *M. luteus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, *Klebsiella planticola* and *C. albicans*, phenazine-1-carboxylic acid (PCA) was most effective as sensitivity was observed at rather low concentrations. PCA also showed high cytotoxic activity against different cancer cells, while 1-hydroxyphenazine did not show any cytotoxic effect.

PCA produced by *P. aureofaciens* Kluyer also showed antibiotic activity against *Mycobacterium tuberculosis* and was named tubermycin B (Haynes et al. 1956). Dimeric compound of PCA produced by *P. fluorescens* 2-79 suppresses soil borne fungal pathogen *Gaeumannomyces graminis* var *tritici* which causes take-all diseases of wheat. Many other phenazine-based compounds have been identified as biocontrol agents for soilborne fungal diseases and approved for commercial production and use (He et al. 2008; Kavitha et al. 2005). Rhamnolipids also exhibited antifungal effect on plant pathogens such as *Colletotrichum orbiculare*, *Magnaporthe grisea*, *Phytophthora capsici*, *Pythium aphanidermatum* and *Rhizoctonia solani* (Rimando and Duke 2006) and also inhibited algae blooms (Wang et al. 2005).

Pseudomonas aurantiaca S-1 produces a strong antimicrobial metabolite with a potential as biopesticide. The antibacterial fraction has a molecular mass 282.8 ($C_{18}H_{36}NO$), and the antifungal activity fraction has a molecular mass 319.3 ($C_{20}H_{31}O_3$). Besides this *P. aurantiaca* S-1 produces indole acetic acid and siderophore that promote plant growth (Mandryk et al. 2007). *Pseudomonas fluorescens* produces mupirocin, an unusual polyketide antibiotic which is currently used in antiseptic creams and ointments. This is effective even against MRSA and variety of other gram-positive and gram-negative bacteria. *Pseudomonas* have emerged as an attractive testing source for new bioactive compounds including antibiotics, bacteriocins, biosurfactants and bacteriolytic enzymes. Besides screening for new bioactive compounds, biotransformation is a valuable strategy to build molecules in the drug discovery programme as well as synthesize compounds whose chemical synthesis is not feasible or is challenging. Members of the genus *Pseudomonas* because of their metabolic versatility are an important group used extensively to generate metabolites in bulk amounts. Biotransformation approaches and synthetic methods in tandem provide a source for generating compounds as a result of reactions such as oxidation, reduction, dealkylation, epoxidation, dehalogenation, oxidative deamination, etc. followed by conjugate reactions with glucuronide conjugates; sulphate conjugates, methylation, acetylation, sulfation, amino acid conjugation and glutathione conjugation; etc. (Ravindran et al. 2012).

8.4.3 Enzymes and Biocatalysis

Members of genus *Pseudomonas* produce a number of extracellular enzymes, and proteases and lipases are particularly important as these find important biotechnological applications. Protease produced in *P. aeruginosa* has properties of stability and activity at alkaline pH, thermostability and resistance to solvents. As such the protease produced by *P. aeruginosa* PD100 is useful in laundry products such as detergents, stain removers, etc. Proteases are also useful as components of pharmaceuticals such as contact lens cleaners and enzymatic debriders. The protease produced by strain PD100 has high collagenolytic activity and has pharmaceutical applications such as (a) cosmetics for skin rejuvenation, wrinkle smoothing and dandruff removal, (b) medicine for fastening wet wounds, (c) leather industry for

manufacture of skin and leather products and (d) biochemistry for isolation of cells from animal tissues. Other applications include removal of blood stain, dehairing of skin and extraction of collagen from skin for collagen replacement therapy, waste treatment and other related uses (Gupta et al. 2002; Najafi et al. 2005).

8.4.3.1 Lipases

Lipases differ greatly with respect to their properties and source, e.g. microorganism, plants and animals. They can catalyse hydrolysis or synthesis of a wide range of substrates. Microbial lipases differ with respect to their substrate specificity and are attractive for industrial applications including detergents, food and flavour, ester and anionic acid derivatives, baking, fine chemicals, bioremediation, hard surface cleaning, leather and paper industry. They are important biocatalysts due to the wide spectrum of substrates, high stability towards extreme temperature, pH and organic solvents and chemo-, regio- and enantioselectivity. The enantioselective and regioselective properties of lipases have been utilized for resolution of chiral drugs, fat modification, synthesis of butter substituents, biofuels and synthesis of personal care products and flavour enhancers (Sharma et al. 2001; Verma et al. 2012).

Two types of enantioselective organic transformations catalysed by lipases are reactions of prochiral substrates and the resolution of racemates. Besides chiral alcohols and esters, other compounds such as cyanohydrins, chlorohydrins, diols, α and β -hydroxyacids, amines, diamines and amino alcohols are produced. Lipases produced by *P. aeruginosa*, *P. fluorescens* and other *Pseudomonas* spp. are important in these biosynthetic reactions. Genetically engineered *P. putida* KT2442 (PSPM01) harbouring toluene dioxygenase (TDO) catalyses asymmetric cis-dihydroxylations of aromatic compounds and can effectively biotransform benzene, toluene or chlorobenzene into respective diols (Ouyang et al. 2007a, b).

Microbial extracellular lipases are usually more thermostable than animal and plant lipases. Lipases from thermophilic bacteria play a significant role in industrial processes as they are thermostable and resistant to chemical denaturation. Majority of the currently available lipases are derived from mesophilic sources and have optimum activity of 35–40 °C. Among several bacteria, *Pseudomonas cepacia* and *Pseudomonas* sp. produce thermostable lipases (Sugihara et al. 1992) (Table 8.3).

Applications Lipases are a biotechnologically important group of enzymes because of their versatility of their applied properties and mass production. They are of commercial importance because of their broad substrate specificity and high stability in organic solvents, and they function under mild conditions and reduced wastes in their use.

Lipases of microbial origin have considerable industrial potential in production of flavour additives (for flavour modification) in food industry (Table 8.4), fine chemicals (synthesis of esters), detergents (hydrolysis of fats), waste matter treatment chemicals (decomposition and removal of oil substances), cosmetics (removal of lipids), pharmaceuticals (digestion of oil and fat foods), leather (removal of lipids)

Table 8.3 Different types of Lipases produced by *Pseudomonas* spp.

Family/ subfamily	Organism	Accession number	Similarity family/ subfamily
1/1 (True lipases)	<i>P. aeruginosa</i>	D50587	100 %
	<i>P. fluorescens</i>	AF031226	95 %
	<i>P. fragi</i>	X14033	40 %
	<i>P. wisconsinensis</i>	W77807	39 %
1/2	<i>P. luteola</i>	AF050153	33/77
1/3	<i>P. fluorescens</i> SIKWI	D11455	14/100
Family			
II	<i>P. aeruginosa</i>	AF005091	35
IV (HSL)	<i>Pseudomonas</i> sp.	B-11/1 AF034088	54
V	<i>P. oleovorans</i>	M58445	100
VI	<i>P. fluorescens</i>	S79600	–

Table 8.4 Applications of enzyme lipase in the food industry

Food industry	Action	Product of application
Dairy foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Development of flavouring agents in milk, cheese and butter
Bakery foods	Flavour improvement	Shelf life propagation
Food dressings	Quality improvement	Mayonnaise, dressings and whippings
Health foods	Transesterification	Health foods
Meat and fish	Flavour development	Meat and fish product, fat removal
Fats and oils	Transesterification, hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono- and diglycerides
Beverages	Improved aroma	Alcoholic beverages, e.g. sake, wine

from animal skins) and medical products (assay of blood glycerides). Lipases from thermophiles are important in industrial processes as they are thermostable and resistant to chemical denaturation (Aravindan et al. 2007).

8.4.3.2 Proteases

Proteases or peptidases are industrially important enzymes and constitute about 60 % of the total enzyme sales. Microbial proteases play an important role in detergent, food, pharmaceutical, textile and leather industry. Application of enzymes in industry is considered eco-friendly and safe, irrespective of the producer organisms. Above that the enzymes are very specific in their mode of action and substrate. Proteases are broadly classified into exo- and endopeptidases depending on their mode of action at the terminus or away from termini. They are also classified as serine proteases, aspartate protease, cystine protease and

metalloproteases depending upon the nature of functional group at the active site. Alkaline proteases are particularly useful in detergent and leather industry as these technologies are ecofriendly and show high activity at alkaline pH (~10) and have broad substrate specificity (Sawant and Nagendran 2014).

A number of proteases have been isolated and characterized from *Pseudomonas*. Proteases of *P. aeruginosa* are mostly involved in pathogenicity of the organism and are responsible for various types of bacterium-host cell interactions. Two alkaline proteases have been isolated and purified from *P. maltophilia*: one is produced near the neutral pH and the other in highly alkaline environment. The organic solvent-tolerant species such as *P. putida*, *P. aeruginosa* and *P. fluorescens* have been considered important for production of variety of enzymes that are organic solvent tolerant. *Pseudomonas aeruginosa* PST01 and PT121 produce proteases that are tolerant to cyclohexane, toluene, ethanol and acetone. The lipolytic enzymes are usually used in nonaqueous medium as these enhance their solubility and flavour product recovery. Lipolytic enzyme produced by *P. aeruginosa* LST03 is stable in the presence of organic solvents such as cyclohexane, toluene, ethanol and acetone (Sardesai and Bhosle 2004; Tang et al. 2010). *Pseudomonas putida* DOT-T1E can withstand 90 % toluene and degrades toluene, while *P. putida* S12 is tolerant to toluene and degrades styrene, octanol and heptanol. The extracellular enzymes of organic solvent-tolerant bacteria are stable, and the problem of microbial contamination is considered lessened. The organic solvent-tolerant bacteria can serve as invaluable agents in catalysing the biotransformations of water-insoluble substances in organic-aqueous biphasic system. For bioconversion cholesterol is usually suspended in systems containing surfactants. However, *P. putida* strain ST-200 which is organic solvent tolerant can degrade cholesterol in biphasic system containing cholesterol dissolved in organic phase and the cells suspended in aqueous phase. Strain ST-200 effectively oxidizes C₃ and C₆ positions to cholesterol by introducing hydroxyl or ketone group in the presence of *p*-xylene and *p*-diphenylmethane, 3:7 v/v, as organic solvent. The enzyme cholesterol oxidase is constitutive and extracellular in nature (Meijnen et al. 2011a, b).

Nonaqueous enzymology has emerged as an important area in biotechnology as their application has been made in chemical processes for synthesis of optically active intermediates, food-related conversions and analysis. Organic solvent-tolerant strains of *Pseudomonas* have potential in bioremediation and degradation of aromatic hydrocarbons. The bacteria can establish and grow to higher densities in soils heavily contaminated with organic solvents such as benzene, xylene, toluene, etc. Potential of *P. putida* DOT-T1E was expanded to include *m*- and *p*-xylene and related hydrocarbons by transfer of TOL plasmid pWWO-km (Ramos et al. 1995; Huertas and Duque 1998).

Large-scale applications of proteases in industry, pharmaceuticals and as therapeutics have been discussed recently (Craik et al. 2011; Li et al. 2013a, b).

8.4.3.3 Amylase

Pseudomonas stutzeri and *Pseudomonas saccharophila* (now *Pelomonas saccharophila*) also produce α -amylase for starch hydrolysis into malto-oligosaccharides, α -amylase of pseudomonads hydrolyses the α -1,4-glycosidic bond by exo-glycolytic cleavage in contrast to the normal endo-glycolytic mode. It is the key enzyme in the production of starch derivatives and widely used in food, textile, paper, detergent, clinical, pharmaceutical and other industrial fields. The G_4 -forming amylases from pseudomonads result in the formation of maltotetraose (G_4), maltopentaose (G_5) and maltohexaose (G_6). G_4 syrup, considered as a partially undigested and unabsorbed substrate in the small intestine, has shown a prebiotic effect by selectively promoting the growth and/or activity of beneficial bacteria, like bifidobacteria in the colon. But they are not utilized by *E. coli* or the *Clostridium* spp. thus favouring the probiotic intestinal flora in the digestive tract and suppressing the formation of putrefactive products (Maalej et al. 2014). Sequence analysis of G_4 and G_5 amylase genes does not show any homology except the COOH terminus responsible for starch binding. G_5 -forming amylase is an important enzyme, and G_5 is used as a reagent for measurement of serum amylase. G_5 is also used as a nutrient for patients suffering from renal failure. *Pseudomonas fluorescens* also produces α -galactosidase which is used for synthesis of hetero-oligosaccharides containing α -galactosyl residues (Hashimoto et al. 1991; Aiyyer 2005).

Apart from these a number of enzymes are used as biocatalysts for the biotransformation reactions for production of industrially important chemicals and pharmaceuticals. Some of these are produced commercially by strains of *Pseudomonas* and are listed in Table 8.5.

Cost-effectiveness of any industrial biotechnology process is important, and an attempt in this direction has been to couple *Pseudomonas*-based processes with that

Table 8.5 Commercial production of enzymes from *Pseudomonas* (Source Rehm 2010)

Enzyme	Industry
Aminopeptidase	DSM, the Netherlands
Aryl alcohol dehydrogenase	Pfizer, USA
Aspartate β -dehydrogenase	Tanabe Seiyaku Co., Japan
Benzaldehyde dehydrogenase	ICI, the UK
Carbamoylase	Dr Vig Medicaments, India
Dehalogenase	AstraZeneca, the UK
Glutaryl amidase	Asahi Kasei Chem. Ind. Co., Japan
Haloalkane dehydrogenase	Daiso Co. Ltd, Japan
Hydantoinase	Dr Vig Medicaments, India
Lipase maltase	Bristol Myers Squibb, the USA
Monooxygenases	Pfizer Inc., the USA Lonza Inc., Switzerland
Nitrile hydratase	DuPont, the USA
Oxidase	DSM, the Netherlands

of renewable feedstock and lignocellulosics from an important component of the renewable feedstock. Lignin is a major structural component of plant biomass and can be subjected to catalytic pyrolysis to generate aromatic compounds which can be utilized for production of valuable products such as cis-cis muconate, a precursor for synthesis of adipic acid (Bu et al. 2011). Enzymatic hydrolysis of cellulose yields glucose and hemicelluloses comprising of pentoses which serve as ready-to-use substrate for industrial biotechnology (Meijnen et al. 2012; Johnson and Beckham 2015).

8.5 Biopolymers

Fluorescent pseudomonads produce two types of biopolymers referred as bioplastics, a polymer comprising of monomer units of polyhydroxyalkanoate esters and the other is the acidic polysaccharide, alginate which has got a high capacity to absorb water and protects the *P. aeruginosa* against phagocytosis by the host cell.

8.5.1 Bioplastics: Polyhydroxyalkanoates

Accumulation of poly[(R)-3-hydroxybutyrate(PHB)] as a reserve material is an important property of aerobic pseudomonads (Stanier et al. 1966; Huisman et al. 1989). *Pseudomonas oleovorans* grown on short-chain aliphatic hydrocarbons develop inclusions of polyester of 3-hydroxyoctanoic acid (PHA). Polymers of polyhydroxyalkanoates form a class of polyesters referred as bioplastics. The crystalline granules in vivo are amorphous and can be derived into bioplastics with properties of biodegradability and biocompatibility for applications in highly specialized areas as biomedical engineering or routine as packaging materials (Khanna and Srivastava 2005; Liu and Chen 2007). Cytoplasmic inclusions of polyalkanoates appear as bright, refractile, insoluble granules representing nearly 50 % of cell mass. Under optimum conditions, it can reach up to more than 80 % of the total cell dry weight. Polyester synthases are the key enzyme for biosynthesis of polyesters. *Pseudomonas putida* accumulate polyhydroxyalkanoates as reserve carbon and energy sources and are a potential source for industrial production. The properties of the final product depend on the monomeric composition. The properties of their elasticity, crystallinity and rigidity are important from the point of view of their use. The short-chain-length polymers such as polyhydroxybutyrate and related polymers show poor physical properties which restrict its use as soft plastics such as packaging films. Thus the composition of the PHA and its physical properties depend on the organism and the carbon source used. All PHAs are polyesters based on stereospecific esterification of hydroxyacyl units. Poly[(R)-3-hydroxybutyrate], i.e. PHB, is most commonly encountered in nature, and its copolymer 3-hydroxyvalerate is less common. The combination of the two was commercialized under the trade name 'Biopol' by Monsanto Bioproducts (Ryu

et al. 1997; Wang and Lee 1997). The medium-chain-length (C_6 – C_{14}) alkanooates were found to be characteristic of fluorescent pseudomonads, *P. putida*, *P. fluorescens*, *P. aeruginosa*, *P. testosteroni* and *P. oleovorans*, which produce PHA consisting of 3-hydroxyoctanoate as a major component. *Pseudomonas putida* KT2440 has been extensively studied, and when grown on *n*-alkane, *n*-alkanoate or *n*-alkanol, the polyhydroxyalkanoate (PHA) produced is a random copolymer containing 3-hydroxyalkanoate units with a carbon chain equivalent to that of the growth substrate as a major component. Other monomer units have either one excess or one or more deficit C_2 pairs. Since the monomer units smaller than hydroxyhexanoate and larger than C_{16} have not been detected, these polymers were referred as medium-chain-length PHA (mcl-PHA). *Pseudomonas putida* LS46 has been isolated from wastewater and accumulates 22–56 % PHA when cultured on glucose, waste vegetable fryer oil or fatty acids. The strain shares 99 % nucleotide identity of 16SrDNA with other strains of *Pseudomonas putida* strains known for plant growth promotion (Sharma et al. 2014).

Pseudomonads can use a variety of substrates for synthesis of mcl-PHA including acetate, propionate and unrelated substrates like glucose as well as long-chain alkanes, alkenes and fatty acids. Formation of PHB would require degradation of the fatty acid to C_2 units and subsequently resynthesis of acetoacetyl-CoA. The process entails considerable energy loss. As a consequence, the *Pseudomonas* strain that grows on long-chain carbon substrates prefers to accumulate mcl-PHA rather than PHB, and even the polymerase is flexible with respect to substrate specificity and allows a wide variety of 3-hydroxyacyl monomers as substrate (Kim et al. 2007a). When grown on substituted carbon sources, the functionalities are often incorporated as such into the mcl-PHA chains. The functionalities that have been introduced include both terminal and nonterminal olefin groups, methyl branches, esterified carboxyl groups and hydroxyl and epoxy groups. Groups introduced in the terminal position include acetoxy, bromine, chlorine, fluorine, phenyl, cyclohexyls, cyano, *p*-cyano, phenoxy and *p*-nitro-phenoxy groups.

mcl-PHAs are potential alternative to petro-based plastics as they are biodegradable and environment-friendly and can be produced on large scale from low cost substrates by microorganisms. Besides, these are suitable for a wide variety of applications.

The short-chain-length (scl) monomers range from C_2 to C_5 carbon molecules as compared to the medium-chain-length (mcl) monomers ranging from C_6 to C_{14} . Scl-PHA have a low glass transition temperature (-30 to -40 °C), a broad melting temperature (around 60 °C) and a low degree of polymerization (usually <100,000 Da). On the contrary the mcl-PHAs show high glass transition temperatures around 0 °C, melting temperatures 180 °C and high molecular mass up to 4 MDa (Williams and Martin 2002a, b; Khanna and Srivastava 2005). Scl-PHAs, e.g. PHB, show high tensile strength, 30–40 MPa, while mcl-PHAs have only 10 MPa. Scl-PHAs show properties similar to classical thermoplasts and as such can compete with polyethylene (PE) or polypropylene (PP) and to some extent the biobased poly(L-lactic acid). The mcl-PHAs are less crystalline and their monomers consist of 6–14 carbon atoms. Sometimes they possess functionalities

that allow prosthetic chemical modifications of mcl-PHA and determine the material properties of the product. They resemble elastomers and latexes that don't become brittle even at low temperature far below the freezing point. They are the interesting biological rubberlike latexes produced by *P. putida*.

Apart from the petrochemical derivatives, fluorescent pseudomonads can use waste/low-cost agriculture-based substrates for sustainable technologies. The long-chain fatty acids from vegetable oil industry such as lauric, myristic, palmitic, stearic, oleic, linoleic and linolenic acids are the low-priced substrates. Crude mixtures are generally found in the waste in vegetable oil industry and are economically attractive. Medium-chain-length poly-3-hydroxyalkanoates are also produced from glucose by strains of *P. putida*. Efforts are afoot to utilize lignocellulosic residues of agricultural industry by converting it into glucose (Bu et al. 2011; Johnson and Beckham 2015).

Members of the genus *Pseudomonas* are natural producers of mcl-PHA as they possess the entire enzymatic machinery to synthesize polyesters from different carbon substrates (Fig. 8.2). They accumulate PHA under conditions of high carbon availability and limitations of nitrogen, oxygen and phosphorus (Madison and Huisman 1999). *Pseudomonas putida* utilize PHAs as reservoir of carbon and energy to cope up with the changing environmental conditions.

Among the various wastes/low-cost feedstocks tested for PHA production including sodium terephthalate produced from PET pyrolysis, raw glycerol, polystyrene pyrolysis oil, animal waste lipids, etc., glucose is the cheapest and most available feedstock for industrial polymer biosynthesis (Agnew and Pfleger 2012).

8.5.1.1 Biosynthesis

The polyhydroxybutyric acid (PHB) accumulation is not the general property of all *Pseudomonas* sp.; however, some species synthesize PHB as cellular reserve food material or polymers of other β -hydroxy organic acids, referred as polyhydroxyalkanoates. PBHs are of short-chain length (scl) and form a brittle thermoplastic material and is commercially produced in combination with its copolymer of 3-hydroxyvalerate as replacement for mineral oil-based plastics. The medium-chain-length (mcl) polymers, mcl-PHA produced by *Pseudomonas*, are suitable for a wide range of applications in packaging industry, medicine, pharmacy, agriculture and food industry or as raw materials for the synthesis of enantiomerically pure chemicals, production of paints, etc. Only one strain, *Pseudomonas* sp. 61-3, is known to produce a blend of PHB and mcl-PHA. The synthesis of PHB involves condensation of two acetyl-CoA molecules by β -ketothiolase (PhaA) leading to the formation of acetoacetyl-CoA which is reduced to (R)-3-hydroxybutyryl-CoA by acetoacetyl-CoA reductase (PhaB). (R)-3-hydroxybutyryl-CoA is the activated precursor of PHA and substrate for polyester synthase (PhaC), and the genes are organized into an operon. In contrast to this, the medium-chain-length (R)-3-hydroxy fatty acids are synthesized by diverting intermediates of fatty acid metabolism to (R)-3-hydroxyacyl-CoA leading to mcl-PHA. If the carbon source is oxidized to acetyl-CoA excluding the β -oxidation pathway, then the fatty acid de novo biosynthesis intermediates are precursors for mcl-PHA biosynthesis in

which the conversion is catalysed by transacylase, PhaG. This is evident from the presence of significant quantities of monomer units with two additional carbon atoms. Furthermore, the fluorescent pseudomonads also produce mcl-PHA from acetate and propionate as well as from gluconate and glucose as growth substrates. The specific transacylase (PhaG) catalyses the transfer of (R)-3-hydroxyacyl moiety of the respective acyl carrier protein (ACP) thioester to coenzyme A (CoA).

The *pha* genes of *P. oleovorans* encoding two PHA synthases and a depolymerase have been found to be homologous to *P. aeruginosa* PAO1. These genes also show homology with *phb* genes of other genera. Granules of PHA also contain a 43 kDa protein as a major component in addition to PHA synthases and depolymerase enzyme, PHA-specific regulator proteins, amphipathic phasin protein and some proteins of unknown functions (Klinke et al. 2000). Only polyester synthase is covalently attached, while all other proteins are non-covalently attached. The PHA granules are bound by phospholipid membrane and are fairly stable. The polyester granules could find applications for protein purification, enzyme immobilization and diagnostics (Rehm 2007). Currently, emphasis is being laid on the production of high-value tailor-made PHA biomaterials for medical applications.

Intracellular levels of NADPH and NADP⁺ were essential for PHA biosynthesis. Therefore, it was considered appropriate to enhance PHA production by manipulation of metabolic pathways for higher NADPH levels. Weakening of the competing β -oxidation pathway in *P. putida* KT2440 by deleting *fadA* and *fadB* genes encoding 3-ketoacyl-CoA thiolase and 3-hydroxyacyl-CoA dehydrogenase significantly increased synthesis of mcl-HPA (Liu and Chen 2007). Overproduction of glucose-6-phosphate dehydrogenase (G6PDH encoded by *zwf*) increases intracellular levels of NADPH and accumulation of PHA by about 41 %. Metabolic engineering of β -oxidation reactions also made it possible to synthesize homopolymers such as poly(3-hydroxyhexanoate), poly(3-hydroxyheptanoate) and poly(3-hydroxydecanoate) as well as a PHA-containing thioester as side chain, which allows desired modifications (Liu et al. 2011; Wang et al. 2011; Escapa et al. 2011). Substrate specificity of type II PHA synthase was modified so that it could accept short-chain-length building blocks for PHA production. High cell densities of *P. putida* also resulted in higher yields, and *P. putida* can be engineered to self-disruption thus reducing the cost of downstream processing (Martinez et al. 2011).

An in silico model was developed based on physiology and large-scale flux mode analysis of metabolism of *P. putida* with a view to predict genetic targets for strain engineering. The suggested target was glucose dehydrogenase (coded by *gcd*) inactivation to prevent the undesirable byproduct formation and excretion. The Δgcd mutant with deleted glucose dehydrogenase was created: *P. putida* KT2440 Δgcd which showed 100 % increase in PHA titre, a 50 % increase of the cellular PHA content and 80 % in PHA yield as compared to the parent strain *P. putida* KT2440. This is an important step for industrial production of mcl-PHA using *P. putida* as a cell factory. Further, in silico metabolic modelling predicted that deletion of glucose dehydrogenase gene *gcd* in combination with overexpression of

pyruvate dehydrogenase subunit gene *acoA* resulted in further improvement in PHA production by 120 % as a consequence of significant change in NADH/NAD⁺ value due to overexpression of pyruvate dehydrogenase complex. Such a strategy is highly promising for evolving superior strains for industrial biotechnology (Palsson 2004; Sohn et al. 2010; Borrero-de Acuña et al. 2014).

Poly- β -hydroxybutyrate is the simplest PHA biopolyester, and besides being biodegradable, these are of lighter mass, thermoplastic and resistant to aberration. *Pseudomonas mandelii* CBST has the advantage and is economical over other psychrotrophs because it uses sucrose as its best carbon source and produces higher cell density. Psychrophilic strain of *P. mandelii* CBS-1 has been reported to produce high levels of poly- β -hydroxybutyrate. The strain grows aerobically to give a high dry cell mass of 29.3 g l⁻¹ and synthesized 22.3 g l⁻¹ of PHB in 48 h (Li et al. 2013a, b; Poblete-Castro et al. 2014).

8.5.1.2 Substrates Used for Biosynthesis of PHA

The large-scale production of PHA is carried out using substrates of high nutritive value such as glucose, starch and edible oils and may account for 50 % of production costs. Attempts are being made to use inexpensive, readily available waste/byproducts of agriculture and industry (Johnson and Beckham 2015). This will help to overcome the problem of disposal of large quantities of agricultural wastes, and the production of biopolymer will be cost-efficient (Nikel et al. 2006). Potential feedstocks for production of PHA by *Pseudomonas* are:

Lignocellulosics—Sugarcane bagasse, rice straw, fruit crop residues, etc. which generate hexoses, pentoses and sugar alcohols.

Potato and starch industry waste—Starch hydrolysis products—maltose, glucose, etc.

Byproduct of biodiesel industry—Glycerol can be used as feedstock.

Oil and fat industry waste—Oils, fats and triacyl-glycerides can be used as feedstock.

Dairy industry waste—Whey—containing lactose and hexoses.

The use of renewable, low-cost substrates provides an efficient alternative feedstock to fossil fuel. Olive oil mill wastewater (OMW) is a rich source of proteins, lipids and polysaccharides. Acidified OMW rich in organic acids has been found suitable for the production of PHA yielding ~90 % PHA/g cell dry mass by a mixed culture (Kourmentza et al. 2015).

8.5.1.3 Recovery and Downstream Processing

Recovery of the final product in relatively pure form is an important aspect of the production bioprocess. PHA is accumulated as intracellular granules and as such involves the separation of the cell biomass from the growth medium, disruption/lysis of the cells and then recovery of the final product from the cell lysate/cell material and finally purification of the end product from the other contaminants.

The economics of the process will depend upon the efficiency of the recovery process and purity of the product. Generally three processes are used.

Direct Extraction of PHA from Biomass The typical halogenated solvents, chloroform, dichloromethane and 1,2-dichloroethane are excellent solvents for scl-PHA and mcl-PHA. After extraction from biomass, it is precipitated by adding ethanol, methanol or acetone as a highly pure product. Since recovery of alcohol by distillation is a high-energy-demanding process and uneconomical, a three-component system of water-chloroform and ethanol has been developed that forms two layers. The lower layer is 95 % chloroform and residues of ethanol and water, and this can be directly used for extraction of PHA; the upper layer contains only negligible amounts of halogenated solvents. Since the halogenated compounds, particularly chloroform, are hazardous to the human health and pollute the environment, it is not a desirable method, and there is a need to develop more safe alternate methods for extraction of PHA.

Digestion of the Non-PHA Cellular Material *Pseudomonas putida* cells rich in PHA granules can be disrupted by a combination of heat shock and treatment with alcalase, sodium dodecyl sulphate and finally EDTA. By this procedure the PHA granules are not affected. Alternatively the biomass is subjected to pretreatment with triton X-100 surfactant and then disrupted in sodium hypochlorite under strong alkaline conditions. For medical application, ultrapure, endotoxin-free PHA can be obtained by sodium hypochlorite treatment. A mechanical method using high pressure homogenizer in the presence of 5 % SDS has been successfully used to yield 98 % PHA. The advantage of this method is that it does not involve the use of any solvent.

Cell Disruption and Centrifugation Physical disruption of cells by suspending in hypotonic solution can be achieved, and the PHA granules can be recovered by low speed centrifugation, sedimentation or filtration. This is a cost-intensive method and suitable for high purity, low volume and high-value products (Martinez et al. 2011).

8.5.1.4 Applications of Bioplastics

Bioplastics find a wide range of applications as packing materials as well as for production of commodity items. Biopol™ products comprising of PHB (polyhydroxybutyrate) and PHB-HV (polyhydroxybutyrate-hydroxyvalerate) are used for shampoo bottles etc. PHA is also used for production of daily commodity items like razors, diapers, hygiene products, cups and dishes, etc. In agriculture these are used for mulching. In paper industry, PHA can be used for latex coating of the paper to improve its strength, gloss, brightness and overall performance. Important applications include:

Application in healthcare systems

Use as materials for different purposes such as packaging

Monomers as chiral intermediates for synthesis of fine chemicals and drugs
Additives as biofuels

Healthcare Systems The property of biocompatibility makes the PHA suitable for a wide range of applications in medical and healthcare systems. PHA can be successfully used as bone implant materials; for tissue engineering as implants, surgical pins, screws, masks and sutures; and as carrier matrices for controlled drug release (Williams and Martin 2002a, b). The production of highly sophisticated surgical articles such as artificial blood vessels, vein valves, bone marrow scaffolds, joints and meniscus regeneration devices, articular cartilage repair devices, nerve guides, ocular cell implants, vein valves, etc. have been reported (Valappil et al. 2006; Bian et al. 2009; Wu et al. 2009).

Developments of implants for children femoral fracture healing and devices under a joint programme of consortium of BRIC (Bio Resorbable Implants for Children) funded by the Government of Australia, industry and universities are under way. The aim is to develop suitable bioplastic implants which when applied can be degraded and absorbed quickly by the tissue so that children don't need another surgery for the removal of implants. For such materials, high purity is a prerequisite; PHAs predominantly produced by gram-negative bacteria carry LPS (lipopolysaccharide) as a contaminant and lead to tissue reactions such as inflammation. LPS can be removed by repeated extraction and precipitation of PHAs, thus a product of required high purity can be obtained. Complete removal of endotoxin (LPS) can be ensured by treatment with H₂O₂, sodium hypochlorite or sodium hydroxide (Sevastianov et al. 2003; Furrer et al. 2007).

The mcl-PHA, poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate) (PHO) produced by *P. putida* GPO1 can be extracted with hexane and 2-propanol as a highly pure product. Indeed, the products required for surgical implants and other medical uses are required to be sterilized. Since mcl-PHA cannot withstand steam sterilization at 140°C, as such they may be subjected to cold sterilization by ethylene oxide or by γ -irradiation. Of course one has to ensure that there is no residue effect of ethylene oxide or any damage to the structure of the material by γ -irradiation. On the contrary polyhydroxybutyrate (PHB) and polyhydroxybutyrate-hydroxyvalerate (PHB-HV) show high melting temperature and can be sterilized by steam. But PHB and PHBV take a long time for in vivo degradation. Another polymer, poly-3-hydroxybutyrate-4-hydroxybutyrate [P (3HB4HB)], shows accelerated biodegradation and has potential for medical uses. USFDA has already approved the use of Tephaflex P4HB as implant material (Williams et al. 2013). With this more PHA-based biomaterials are expected in clinical trials as bioimplants (<http://www.tepha.com>).

Because of their property of biodegradability, biocompatibility and degradation by surface erosion, PHB and PHBV have been found suitable as carriers for the sustained release of the drug. PHA granule-binding protein, PhaP, is able to bind to hydrophobic polymers. The system consisting of PHA nanoparticles, PhaP and ligands fused to PhaP has been used for target-specific drug delivery (Yao et al. 2008).

Packaging Materials Packaging occupies 41 % of the total plastic usage and occupies the top position. Out of this 20 % is used by the food industry. Required properties of good packaging materials are permeability (gas and vapour), sealing, resistance to chemicals, UV and visible light, transparency, mechanical properties, machinability, etc. The conventional petro-based plastics possess all these properties except sustainability which bioplastics offer. The bioplastics are compostable and thus reduce the landfill waste. Various types of blends and combinations are now available as bioplastics and are being produced commercially under the names 'Eco PLA', 'Ecoflex', 'Ecovio', 'Sorona', etc.

Initially, bioplastics were used for manufacture of packaging materials such as milk cartons and films, moisture barriers in nappies and sanitary towels, pens, covers for cardboards and papers, combs, bullet cosmetic containers, shampoo bottles, etc. Now their applications include more specialized products such as pens, containers, golf tees, personal hygiene materials (such as diapers), bags and lids or tubs for thermos-formed articles, films, flavour delivery agents in foods, dairy, cream substitute, fibre and other materials suitable for composting. They also find application as heat-sensitive adhesive materials, latex and smart gels for specialized purposes (Tanaka et al. 2007).

Smart Materials PHAs harbouring special building blocks can yield smart functional materials suitable for niches requiring heat-sensitive adhesives, latex materials or smart gels (Chen 2009). Of special interest is PHA as basic material connected by linkers with biologically active substances, e.g. coating of surfaces of ships/boats by mcl-PHA-based matrix linked with zosteric acid is used to protect it from biofouling (Hany et al. 2004; Lee and Na 2013).

Future Material Applications Bioplastic production has its limitations such as high cost of production and product safety. Production of composites and blends will overcome or minimize drawbacks in the properties that limit their applications. Future applications include areas of medical/biomedical applications and development of devices, artificial organs and therapeutic uses (Wu et al. 2008; Bian et al. 2009). These also include cardiovascular products (pericardial and arterial septal repair patches, vascular grafts, cardiovascular stents and heart valves), and products for orthopaedic and urology procedures and management of wounds (Wang et al. 2008; Yang et al. 2010). Carrier materials and degradable matrices can be produced from PHA for the desired release of fertilizers, pesticides, plant growth hormones in agriculture, release of drugs and antibiotics in pharmaceutical sciences and release of dye stuff and flavours in the food industry. Tailoring PHA composition with unique properties will be useful for production of products with ultra high molecular weight and desirable properties of mechanical strength, copolymerization with other molecules, biocompatibility and degradation (Chen and Wu 2005) PHAs also find application as metabolic regulators and enhance robustness of industrial microorganism to tolerate stress of fermentation conditions. The PHA surface-binding proteins can be purified as they are useful as specific drug

delivery systems. The cost-effectiveness of the production of bioplastics shall, however, remain a priority for the scientists and technologists.

Chiral Compounds and Drugs Polyhydroxyalkanoates can be hydrolysed into monomers which are a rich source of optically pure chiral compounds, R(-) configured bifunctional hydroxyl acids that can serve as starting material (synthons) for syntheses of fine chemicals, drugs, hormones, pheromones, vitamins, antibiotics and pharmacologically bioactive compounds. These follow-up compounds possess higher market value than PHA as such. Efficient methods are being developed for the activation of the hydrolytic enzymes for depolymerization of biopolymers within the cells (Ren et al. 2009; Chen and Wu 2005). Monomers formed as a result of hydrolysis of PHA are a rich source of chiral, optically pure R(-)-configured bifunctional hydroxyl acids which can be used for synthesis of fine chemicals such as pheromones, aromatics, vitamins and antibiotic or other bioactive compounds. 3-Hydroxybutyric acid and its oligomers have therapeutic properties, by promoting cell growth and retarding necrotic cell death. Sodium salts of butyric acids (D-3HB, DL-3HB, 3HBME) are derived from ketone produced in animals and human beings and considered for tissue engineering applications by inhibiting cell apoptosis and elevating cytosolic Ca^{+} concentration.

Learning and memory require energy-demanding cellular processes and can be enhanced by supplementing metabolic substrates. Monomers of the PHB have been reported to enhance the metabolic activity of neurological cells in cell cultures.

Currently, 3-hydroxyalkanoate methyl esters are considered as effective drugs in Morbus Alzheimer therapy (Cao and Zhang 2012). A variety of such chiral compounds is accessible from PHA. Such compounds show higher economic value than PHA itself, but depolymerization of PHA and recovery of the product are highly energy-intensive processes. Methods are being developed for triggering in vivo depolymerization of the intracellular PHA.

Biofuels

Recently, esters of 3-hydroxybutyrate (3HBME) and mcl 3-hydroxyalkanoate methyl ester (3HAME) obtained from esterification of PHB and mcl PHA have been reported for use as biofuels. These can be used to supplement biofuels for enhancing the calorific value of fuels (Zhang et al. 2009).

8.5.2 Alginate

Alginate is a natural polysaccharide exhibiting excellent biocompatibility and biodegradability properties, having many different applications in the field of biomedicine. Alginate is applicable as three-dimensional scaffolding materials such as hydrogels, microspheres, microcapsules, sponges, foams and fibres. Alginate-based dressings have been designed to promote wound healing by maintaining moist occlusive environment. Alginate-based biomaterials are also

used for cartilage repair and tissue engineering by injectable hydrogels and solid and gel spheres for cartilage regeneration. Alginate-based hollow microcapsules have great potential as drug delivery systems and cell carriers for tissue engineering. Alginate can be easily modified via chemical and physical reactions to obtain derivatives having various structures, properties, functions and applications. Alginates as gel-forming polymers have a variety of industrial applications such as stabilizing, thickening and gelling agents in food production or for immobilization of cells for use in pharmaceutical and biotechnological industries (Pawar and Edgar 2012). Alginates are produced by *P. aeruginosa* and some other species including *Azotobacter* sp. and brown seaweed algae. Alginates are water-soluble exopolysaccharides comprising of a family of non-repeating unbranched copolymers consisting of (1-4)-linked β -D-mannuronic acid and its C₅ epimer α -L-guluronic acid. These co-monomers are distributed in blocks of continuous mannuronate residues (M blocks) and guluronate residues (G blocks) or alternating (MG blocks) residues. The alginates produced by *Pseudomonas* don't contain G blocks, and the mannuronate residues of alginates of bacterial origin are acetylated to a varying degree at position O-2 and/or O-3. The physicochemical properties of alginate depend upon the sequence of the co-monomers and the extent of acetylation; this has a bearing on their material properties. Properties of biocompatibility self-assembly and possibility of designing suitable biopolymers have enhanced their applicability in medicine as scaffolding for tissue engineering, beads for drug delivery and blends with other biopolymers to enhance material properties. Mannuronic acid derived from engineered polymannuronate produced by bacteria has been found to be an immunosuppressive, anti-inflammatory drug in the treatment of multiple sclerosis, arthritis and nephritis. Such applications require highly purified and structurally defined molecules (Remminghorst and Rehm 2006).

In *P. aeruginosa* a set of 24 genes has been identified to be directly involved in alginate biosynthesis. Alginate acts as a virulence factor for establishment of infection by biofilm formation. Some *alg* genes encode proteins that are not exclusively required for alginate biosynthesis, e.g. regulator proteins and enzymes catalysing cytosolic biosynthetic steps. All functional genes involved in the synthesis of precursor guanosine-diphosphate (GDP)-mannuronic acid have been characterized. Fructose-6-phosphate has been identified as the first alginate precursor derived from ED pathway. Enzyme activities leading to alginate precursor, GDP-mannuronic acid, have been identified. The precursor biosynthesis requires that the central metabolite fructose-6-phosphate is converted by various enzymatic biosynthesis steps into GDP-mannuronic acid, the immediate precursor of alginate. Pyruvate derived from sugar oxidation is channelized to citric acid cycle. Alginate is also competitive with PHA for acetyl-CoA requirement (Hoffman and Rehm 2005; a Muhammadi and Ahmad 2007). The GDP-mannose dehydrogenase (Alg D) plays a key role in alginate biosynthesis by catalysing oxidation of GDP-mannose to GDP-mannuronic acid. The *algD* gene is located downstream next to promoter and its expression is under tight control. The enzyme phosphomannose isomerase/guanosine diphosphomannose pyrophosphorylase (PMI-GMP) (AlgA) protein is a bifunctional protein catalysing 1st and 3rd step of alginate biosynthesis. The second

step is catalysed by phosphomannomutase (AlgC) resulting in the formation of mannose-1-phosphate. AlgC also exhibited phosphoglucomutase activity and is involved in the biosynthesis of rhamnolipid and lipopolysaccharide in addition to alginate biosynthesis. The GMP activity of AlgA converts mannose-1-phosphate to GDP-mannose with simultaneous hydrolysis of guanosine triphosphate (GTP). Enzyme guanosine diphosphomannose dehydrogenase catalyses oxidation of GDP-mannose to GDP-mannuronic acid which is the direct precursor for polymerization (Rehm 2006). Alginate polymerase is a complex protein in the cytoplasmic membrane which catalyses polymerization of GDP-mannuronic acid to alginate. AlgE forms an alginate-specific pore which enables export of nascent alginate through the outer membrane.

Three alginate enzymes, the transacetylase, C-5-mannuronam epimerase and a lyase have been identified as alginate-modifying enzymes for the production of tailor-made alginates by fermentation. Enzyme C-5 mannuronan epimerase which catalyses post-polymerization conversion of M residue to G residue is of particular interest. Genes *algI*, *algJ* and *algF* code for transacetylase and other proteins involved in transacetylation, and the epimerase (AlgG) and lyase (AlgL) are encoded by genes *algE* and *algL*, respectively. Lyase is an alginate biosynthesis enzyme and functions as an editing protein controlling molecular weight of alginate. As properties of the polymer vary widely with the monomer sugar sequence, microbial systems offer exciting opportunities to produce tailor-made polymers with desired properties. Further understanding enzymes of the alginate biosynthesis will pave way for the biosynthesis of tailor-made alginate using techniques of metabolic engineering (Ertesvag et al. 1999; Ruffing and Chen 2006; Sun and Tan 2013).

8.6 Biosurfactants

Surfactants are amphiphilic compounds which lower tension between two surfaces and play an important role in our daily life as they are used as detergents, paints, emulsifiers, adhesives, inks, defoaming agents, soil decontamination, pharmaceuticals and agrochemical formulations such as pesticides, herbicides, etc. for their proper dispersal and effective use. By and large the surfactants are produced by chemical synthesis and are derived from petrochemical feedstock thus causing stress on the limited natural resources and the environment. In contrast to this, biosurfactants produced by microorganisms are biodegradable and environment-friendly, low in aquatic toxicity and produced from renewable, biocompatible and digestible resources thus making them suitable for use in pharmaceutical and food industry (Reis et al. 2013).

A variety of biosurfactants are produced by different microorganisms which act at the interface by reducing the surface tension. Glycolipids form an important class of biosurfactants and comprise of a carbohydrate hydrophilic polar head connected to hydrophobic long-chain aliphatic acids or hydroxy aliphatic acids. Members of the genus *Pseudomonas* are predominant producers of rhamnolipids (RL), the

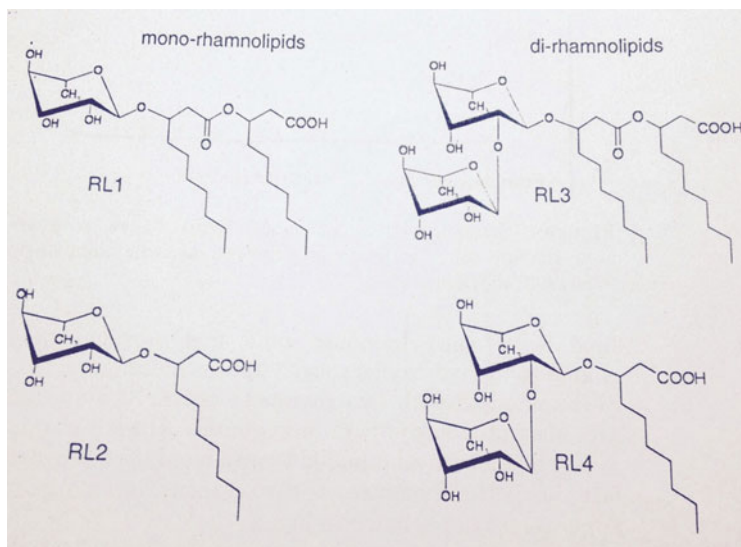


Fig. 8.4 Structure of the four types of rhamnolipids produced by *P. aeruginosa*

rhamnose-based glycolipids. *Pseudomonas aeruginosa* has been recognized as the primary producer of rhamnolipids, though lately other species of *Pseudomonas* and other genera have been identified to produce rhamnolipids. *Pseudomonas aeruginosa* produces a mixture of four different types of rhamnolipids in batch culture, i.e. di-rhamno-di-lipid 67 %, mono-rhamno-di-lipid 22 %, di-rhamno-mono-lipid 9 % and mono-rhamno-mono-lipid less than 3 % (Arino et al. 1996; Nitschke et al. 2005) (Fig. 8.4). A total of 58 congeners of rhamnolipids produced by different bacteria have been identified.

8.6.1 Rhamnolipids

Rhamnolipids are the main class of biosurfactants produced by *P. aeruginosa*, *Burkholderia* sp. and some other species of *Pseudomonas*. Two classes of rhamnolipids are mono- and di-rhamnolipids depending upon the number of rhamnose sugar moieties linked to β -hydroxy fatty acid chains (Perfumo et al. 2006; Raza et al. 2009). They show high surface activity and find application in biomedical sciences, as they display antibacterial, antifungal, antiviral and antiadhesive properties, preparation of nanoparticles (Palanisamy and Raicur 2009) and microemulsions (Nguyen and Sabatini 2009; Costa et al. 2010).

Among the various biosurfactants produced by different microorganisms, the rhamnolipids hold potential for commercial production and are considered next-generation biosurfactants. Studies are now underway to enhance their productivity by optimization of culture medium and physical and chemical parameters. Genetic manipulation and metabolic engineering strategies are also being employed to

enhance the metabolic fluxes towards the product formation (Vermuri and Aristidou 2005).

Biotechnological production of rhamnolipids is rather expensive and range between 200 (20 % solution) and 6000 US\$/kg⁻¹ (98 % pure) compared to synthetic surfactants 1–3 US\$/kg⁻¹ (Leitermann et al. 2010). In the USA, one company, Rhamnolipid Inc. in St. Petersburg, is producing rhamnolipid commercially. For competitive marketing of rhamnolipids, it is necessary to reduce their production costs and increase production rates (Reis et al. 2011).

As already pointed out, although *P. aeruginosa* are the primary rhamnolipid producers, several other species of *Pseudomonas* and related genus *Burkholderia* produce rhamnolipids. A number of congeners of these with varying chain lengths and hydroxylation of carbon have been identified from a variety of soil microorganisms. Thus, overall the rhamnolipids represent glycosides comprising of glycan part (rhamnose moiety) and aglycan part (lipid moiety) linked through o-glycosidic bond. The glycan part is composed of one (mono-RL) or two (di-RLs) rhamnose moieties linked through α -1,2-glycosidic linkage. The aglycan part is one or two β -hydroxy fatty acid chains (saturated, mono- or polyunsaturated chains) varying between C₈ and C₁₆ linked through ester bond formed between β -hydroxy group of distal chain and COOH group of the proximal chain. Detailed review of the subject has been presented by Abdel-Mawgoud et al. (2011). Modification in the glycan and aglycan parts results in different homologs, and presently about 60 different homologs produced by bacteria have been identified (Déziel et al. 1999, 2000).

8.6.1.1 Bacteria-Producing Rhamnolipids (RLs)

Apart from *P. aeruginosa*, other species of *Pseudomonas* have been reported to produce rhamnolipids (Gunther et al. 2005, 2006; Onbasli and Aslim 2009). Strains of *Pseudomonas alcaligenes*, *P. cepacia*, *P. chlororaphis*, *P. clemancea*, *P. putida*, *P. fluorescens*, *P. plantarii* and *P. stutzeri* along with genera *Acinetobacter*, *Enterobacter* and *Pantoea* among Gamma-proteobacteria and the genus *Burkholderia* of Betaproteobacteria besides some *Bacilli* and *Actinobacteria* have been reported to produce rhamnolipids. Some of the important types of rhamnolipids produced by *Pseudomonas* are listed in Table 8.6.

RLs have a multifunctional physiological role among the producer organisms. Important among these are they (1) enhance solubility of poorly soluble compounds and thus promote their uptake and degradation, (2) RLs may act as immune modulators and virulence factor, (3) RLs also display a wide range antimicrobial properties against a number of gram-positive and gram-negative bacteria and some plant pathogenic fungi, and (4) surface moiety and biofilm development (Müller and Hausmann 2011; Müller et al. 2012).

8.6.1.2 Biosynthesis

The main types of RLs produced by *P. aeruginosa* are RL-1, the mono-rhamnolipid, rhamnosyl- β -hydroxydecanoyl- β -hydroxydecanoate (Rha-C₁₀-C₁₀) and RL-3, di-rhamnolipid, rhamnosyl-rhamnosyl- β -hydroxydecanoyl- β -hydroxydecanoate

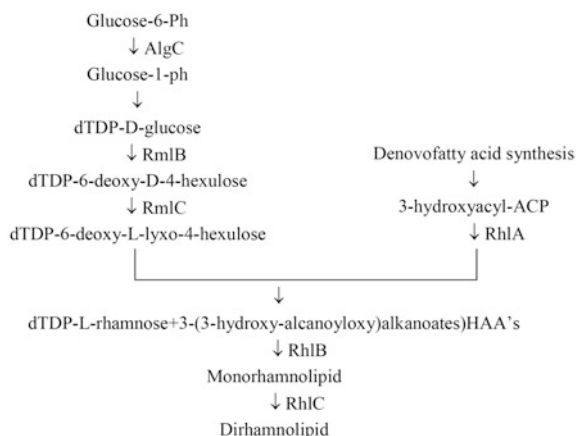
Table 8.6 Important *Pseudomonas* strains producing rhamnolipids

1.	<i>P. aeruginosa</i> various strains	Mixture of dirhamnolipid and monorhamnolipid	References
2.	<i>P. chlororaphis</i>	Rha-C ₁₀ -C _{12:1}	Dubeau et al. (2009)
		Rha-C ₁₀ -C ₁₂	
		Rha-C ₁₀ -C ₁₄	
		Rha-C ₁₀ -C ₁₆	
3.	<i>P. putida</i>	-C _{18:2} , -C ₂₀ , C ₂₂	Martinez-Toledo et al. (2006)
4.	<i>Burkholderia glumae</i>	Rha-Rha-C ₁₀ -C ₁₀	Pajarron et al. (1993)
		Rha-Rha-C ₁₄ -C ₁₄	
5.	<i>B. plantarii</i>	Rha-Rha- C ₁₂ - C ₁₄	Andra et al. (2000)
		Rha-Rha- C ₁₄ - C ₁₂	Hörmann et al. (2010)
		Rha-Rha- C ₁₄ - C ₁₄	Andra et al. (2000)
6.	<i>B. pseudomallei</i>	Rha-Rha-C ₁₄ -C ₁₄	Dubeau et al. (2009)
		Rha-Rha-C ₁₂ -C ₁₄	
		Rha-Rha-C ₁₄ -C ₁₆	

(Rha-Rha-C₁₀-C₁₀), while RL-2 (Rha-C₁₀) and RL-4 (Rha-Rha-C₁₀) are produced in small amounts (Déziel et al. 2000; Dubeau et al. 2009). However, 25 rhamnolipid congeners with varying chain length and saturation have been identified in *P. aeruginosa*. The precursors for rhamnolipid synthesis are derived from the central metabolism of glucose and the hydroxyl fatty acids from fatty acid de novo synthesis starting with acetyl-CoA. RL synthesis by *P. aeruginosa* proceeds in three sequential steps: (1) protein RhlA (encoded by gene *rhlA*) catalyses the synthesis of fatty acid dimer moiety of rhamnolipids and free 3-(3-hydroxyalkanoyloxy) alkanolic acid (HAA) precursor (Deziel et al. 2003); (2) a membrane-bound RhlB, rhamnosyl-transferase (encoded by *rhlB* gene) uses the dTDP-L-rhamnose and HAA molecule as precursors yielding mono-RL (Ochsner et al. 1994); and (3) the RhlC, rhamnosyl-transferase catalyses the rhamnosylation of mono-rhamnolipid to di-rhamnolipids (Rahim et al. 2001). Recent studies suggest that RhlA is responsible for diverting the β -hydroxydecanoyl-ACP intermediate from the bacterial fatty acid synthesis system (FASII cycle), reviewed by Lopez-Lara and Geiger (2010), by directly competing with FabA and FabI for this intermediate (Zhu and Rock 2008). In addition, RhlA is the only protein required to convert two molecules of β -hydroxyacyl-ACP into an HAA. This is an important step as it provides substrate for RhlAB (Reis et al. 2011).

Rhamnose is a deoxy-hexose sugar widely found in bacteria and plants, but not in humans. The activated L-rhamnose is derived from a glucose scaffold in four sequential steps, yielding deoxythymidine di-phospho (dTDP)-L-rhamnose (Fig. 8.5).

The first enzyme in the dTDP-L-rhamnose pathway is glucose-1-phosphate thymidyltransferase (RmlA) which catalyses the transfer of a thymidylmonophosphate nucleotide to glucose-1-phosphate. RmlA is a homotetramer and is allosterically regulated by dTDP-L-rhamnose, the end product of the pathway

Fig. 8.5 Synthesis of rhamnolipid in *P. aeruginosa*

(Blankenfeldt et al. 2000), thus affecting all the downstream reactions of the pathway. The second enzyme, dTDP-D-glucose 4,6-dehydratase (RmlB), catalyses an oxidation of the C4 hydroxyl group of the D-glucose residue, followed by dehydration, leading to the formation of dTDP-4-keto-6-deoxy-D-glucose (Allard et al. 2001). The third enzyme, dTDP-4-keto-6-deoxy-D-glucose 3,5-epimerase (RmlC), catalyses a double epimerization reaction at the C3 and C5 positions of the 4-keto-6-deoxy-D-glucose ring. Finally, dTDP-4-keto-6-deoxy-L-mannose reductase (RmlD) reduces the C4 keto group of dTDP-L-rhamnose (Graninger et al. 1999). All four enzyme genes are organized as a single operon in *P. aeruginosa*, called *rmlBDAC*.

The biosynthesis of rhamnolipid sugar moiety proceeds in the same way as the production of LPS as well as the synthesis of exopolysaccharide alginate. Rhamnolipid biosynthesis is regulated by complex regulatory mechanism and quorum-sensing response which also controls virulence in *P. aeruginosa*. The main components of a quorum-sensing system are the QS signal synthase, the signal receptor (regulatory protein) and the signal molecule (Williams 2007; Williams and Camara 2009). The synthases LasI and RhlI produce the homoserine lactones (HSL) 3OC12-HSL and C4-HSL, respectively, which complex with their correspondent transcriptional regulators, LasR and RhlR, to modulate the transcription of 5–10 % of *P. aeruginosa* genome. Genes *rhlA* and *rhlB* are arranged in an operon (*rhlAB*) and are clustered with *rhlR* and *rhlI* involved in transcriptional and posttranscriptional regulation through quorum-sensing mechanism. Gene *rhlC* is located elsewhere on the chromosome (Reis et al. 2011). In contrast to *P. aeruginosa*, *P. chlororaphis* produces only monorhamnose RLs which probably lacks homolog of gene *rhlC*, encoding rhamnosyltransferase required for synthesis of di-RLs (Gunther et al. 2005). The dTDP-L-rhamnose represents the rhamnose moiety and plays a key role in the biosynthesis of rhamnolipids by regulating the activity of the first enzyme, RmlA (glucose-1-phosphate-thymidyltransferase), of the pathway. dTDP-L-rhamnose is also channelized to structures such as extra-cellular polysaccharide (EPS) and lipopolysaccharide (LPS).

8.6.1.3 Production of Rhamnolipids

Screening of a large number of species and strains has resulted in selection of two *P. aeruginosa* strains DSM 2874 and DSM 7108 for production studies. Strain 2874 produces RL3 and RLR1 in the ratio of 3:1, while DS7108 produces both these rhamnolipids in more or less equal amounts. Though *P. aeruginosa* are good producers, the difficulty is that *P. aeruginosa* are widely distributed and are pathogenic causing nosocomial and respiratory infections particularly in patients with compromised immune system (Walter et al. 2010). The complexity of *P. aeruginosa* QSR, the transcriptional regulatory network involved in the production of rhamnolipids and other virulence-associated traits make the isolation of hyper-producing strains rather different. As an alternative, rhamnolipid production in heterologous hosts by cloning *rhlAB* rhamnosyltransferase with *P. fluorescens*, *P. putida*, *P. oleovorans* and *E. coli* was attempted (Ochsner et al. 1995). *Pseudomonas putida* showed best results with a production of 60 mg l⁻¹ of rhamnolipids. Cha et al. (2008) by cloning *rhlAB* along with the regulatory genes *rhlRI* achieved a level of 7.2 g l⁻¹ of rhamnolipid production by the recombinant *P. putida* *rhlABRI*.

Various types of cultivation strategies such as nature of substrate used, cultural conditions of temperature, pH, antifoaming agents, and bioreactor types have been used to enhance productivity of rhamnolipids (Guerra-Santos et al. 1984). *Pseudomonas aeruginosa* DSM7108 showed a yield of 20–30 g l⁻¹ on plant oil. The specific productivity was similar for all plant oils used and ranged around 7 mg g⁻¹ DBM and h (DBM is the dry biomass concentration). Glycerol, a byproduct of biodiesel production, is a suitable feedstock (Rahman et al. 2002). The subject of production of rhamnolipids and downstream processing has been reviewed elsewhere (Lee et al. 2004; Chen et al. 2007; Walter et al. 2010; Wang et al. 2007; Mukherjee et al. 2006; Benincasa 2007; Fakruddin 2012; Banat et al. 2010).

There has been an increase in the interest of rhamnolipids for cleaning, food, cosmetics, pharmaceuticals and environmental applications as these are sustainable and environmentally compatible. However, two major impediments are the predominant producer organism, *P. aeruginosa*, being pathogenic to human beings and the high cost of production as compared to conventional petrochemical-based detergents. Thus, there is a need to widen the scope of microorganisms and screen for non-pathogenic and industrially safe organisms and develop processes that are economically viable, for example, the use of cheap raw materials and efficient technologies (Maier and Soberón-Chávez 2000; Setoodeh et al. 2014). For screening we can look for the homologs of genes *rhlA*, *rhlB* and *rhlC* and the manipulation of their regulatory processes may result in higher yields. Downstream processing and purification also involve high cost. Thus one can look for products with lower purity for use as cleaning agents, agriculture and environmental applications and go for higher purity for products that are used in high-end products such as food, cosmetics and pharmaceuticals. Development of high viscosity in fermentation broth due to synthesis of side products such as alginate also adversely affects downstream processing. All these problems need to be handled for an economically viable industrial process (Thanomsub et al. 2006).

8.6.2 Applications of Biosurfactants

Biosurfactants have potential use in industry such as lubrication, wetting, softening, fixing dyes, emulsions, stabilizing dispersions, foaming and prevention of foaming, as well as in food, biomedical and pharmaceutical industry, agriculture and environmental bioremediation. Important applications of rhamnolipids are as follows.

8.6.2.1 Food Industry

In food industry biosurfactants are used as emulsifiers, foaming and wetting agents, solubilizers, anti-adhesives and antimicrobial agents (Singh and Cameotra 2004). Rhamnolipids are important agents used to control consistency of bakery and ice cream formulations and improve the shelf life and stabilize aerated systems and overall texture of foods. Furaneol, a high-quality flavour agent, is synthesized from L-rhamnose obtained by hydrolysis of rhamnolipids produced by *P. aeruginosa* (Freire et al. 2010).

8.6.2.2 Medicine

Biosurfactants are useful in a number of ways in medicine and pharmaceutical industry by virtue of their inherent properties (Magalhaes and Nitschke 2013; Fracchia et al. 2015).

Antimicrobial Activity Rhamnolipids are effective bacteriostatic agents resulting in lysis of the cells as they disrupt the cell membrane. Lipopeptides possess strong antimicrobial activity, e.g. surfactin shows antimicrobial, antimycoplasma, antiviral and haemolytic activities and is effective as an antimicrobial agent against gram-positive bacteria, viz. *Bacillus cereus*, *Micrococcus luteus*, *Staphylococcus aureus* and *Listeria monocytogenes* infections. RLs also show significant inhibitory activity against gram-negative species such as *Serratia marcescens*, *Enterobacter aerogenes*, *Klebsiella pneumonia* and a number of fungi. Lipopeptide surfactants, putisolvin I and II, produced by *P. putida* inhibit biofilm formation as well as breakdown of existing biofilms (Kuiper et al. 2004). Thus they are useful as antiadhesive agents in the treatment of many diseases as well as therapeutic and probiotic agent. Bacterial lipopeptides are nontoxic and non-pyrogenic and thus are useful in immunomodulators (adjuvants) when mixed with conventional antigens. Rhamnolipid produced by *P. aeruginosa* B-1 is effective against *B. cereus*, *S. aureus*, *M. luteus*, *Mucor miehei* and *N. crassa* (Nitschke et al. 2009). *Pseudomonas libanensis* strain M-93 produces a cyclic lipopeptide, viscosin with antimicrobial properties (Saini et al. 2008). RLs have also been reported with antiviral, algicidal, mycoplasmicidal and antiamoebic properties (Rodrigues and Teixeira 2008; Abdel-Mawgoud et al. 2010).

Biosurfactants are also used in gene delivery and nanobiotechnology as liposomes for the delivery of foreign genes into mammalian cells. Mannosylerythritol (MEL) containing liposome has been found useful in lipofection (Gharaei-Fathabad 2011). MELs are self-assembling, interact with carbohydrate ligands and

show high affinity for immunoglobulins G and M and lectins. Rhamnolipids have been used for generating silver nanoparticles with antimicrobial activity, nickel oxide nanoparticles, ZnS nanoparticles and microemulsions (Banno et al. 2012).

Di-rhamnolipids have been reported to show anticancer activity. *Pseudomonas aeruginosa* M14808 produces a rhamnolipid comprising of mono-rhamnolipid Rha-C10-C10 and di-rhamnolipid Rha-Rha-C10-C10 as major components. The di-rhamnolipid fraction shows significant antiproliferative activity against human cancer MCF-7 and H460 cell lines (Zhao et al. 2013).

8.6.2.3 Cosmetic Industry

Rhamnolipids are good moisturizing agents and do not cause skin irritability and toxicity, thus are useful in wound healing with reduced fibrosis, cure of burn-shock and wrinkle treatment. Products like acne pads, antidandruff, antiwrinkle and antiageing products and skincare formulations contain rhamnolipids (Piljac and Piljac 2007).

8.6.2.4 Detergents and Cleaning Agents

Currently used laundry detergents are by and large chemically synthesized and exert toxic effects to living organisms. To overcome the environmental hazards and risks, biosurfactant-based detergents have been introduced as they are good emulsifiers and surface active agents and are used in laundry products, shampoos and soaps (Parry et al. 2013). Particularly, cyclic lipopeptides (CLPs) are used, as these are stable over a wide range of pH (7.0–12.0) and high temperature (Mukherjee 2007).

8.6.2.5 Biosurfactants in Pollution Control and Bioremediation

Due to their ability to emulsify hydrocarbon-water mixtures, biosurfactants are an important tool for enhancement of hydrocarbon degradation in nature, thus they are useful in degradation of hydrophobic contaminants. Crude oils have very low water solubility, high adsorption on the soil matrix and are a barrier against microbial degradation. Biosurfactants are used to reduce tension at the hydrocarbon-water interface thus enhancing their solubility, mobility and bioavailability for microbial degradation. Rhamnolipids were effective in removing polycyclic aromatic hydrocarbons (PAHs) and pentaphenol from soil with 60–80 % removal efficiency. Purified rhamnolipids were applied for the removal of oil from contaminated sandy soils and facilitate bioremediation of oil spills (Rahman et al. 2003; Cameotra and Makkar 2010).

8.6.2.6 Surfactants in Petroleum Industry

Biosurfactant-producing microorganisms along with nutrients are injected into oil wells to enhance oil recovery. An increase of 30–200 % in oil recovery has been reported by injection of biosurfactants and bacteria such as *P. aeruginosa*, *Xanthomonas campestris* and *Bacillus licheniformis* along with nutrients (Singh et al. 2008). Biosurfactants have potential application for oil recovery from petroleum tank bottom sludges, thus facilitating transport of heavy crude oil through

pipelines. Rhamnolipids are specially used for oil recovery of soaked oil from used oil sorbents with up to 95 % recovery (Wei et al. 2005; Whang et al. 2008).

8.6.2.7 Application in Agriculture

Biosurfactants help the soil microflora to adsorb to the soil particles occupied by pollutants, thus decreasing the diffusion pathway between the site of absorption and site of bio-uptake by microorganisms. The microorganisms gain access to the pollutant either by biosurfactant-facilitated processes (emulsification and solubility) or by adhering to the hydrocarbon droplet as a result of enhanced hydrophobicity. Biosurfactant-mediated wettability of heavy soils also ensures even distribution of fertilizers in soil and nutrient availability. They also prevent caking during storage. Rhamnolipids mostly produced by *Pseudomonas* also possess antimicrobial activity, and at the same time they don't show any adverse effect to human beings and are useful in control of soilborne diseases (Sachdev and Cameotra 2013).

Besides glycolipids other classes of biosurfactants produced by microorganisms are phospholipids and fatty acids, lipopeptides and lipoproteins, polymeric biosurfactants and particulate biosurfactants. Among these lipopeptide biosurfactants, putisolvin I and II produced by *P. putida* have also been reported to possess pesticidal activity by acting against biofilm formation and facilitating swarming movement. Lipopeptide biosurfactants produced by several other bacteria also exhibit insecticidal activity against *Drosophila melanogaster* and has potential as a biopesticide (Mulligan 2005). Zonix, a commercially available rhamnolipid biosurfactants mixture, is used as a contact fungicide for control of downy mildews (*Pythium* sp. and *Phytophthora* sp.) on horticultural and agricultural crops (Approved by USEPA).

8.7 Natural Products by Heterologous Gene Expression

Heterologous gene expression enables biotechnological production of a variety of valuable natural products. The genetically engineered organisms are characterized by easy handling as compared to the natural producers. They are also free from the natural regulatory mechanisms and thus are hyper producers; further, the use of strain like *P. putida* KT2440 which has been certified as safe (GRAS) allows their use at the laboratory scale as well as industrial scale. Over the recent times *Pseudomonas putida* has emerged as an efficient work horse for genetic manipulation and has virtually been domesticated by using techniques of synthetic biology (Lee et al. 2012a, b; Nikel et al. 2014; Loeschcke and Thies 2015)

Synthesis of a number of natural products that are extensively used in medicine and agriculture involves a complex process using multifunctional megasynthases. Expression of such large DNA segments in suitable hosts holds promise, and a versatile organism as *Pseudomonas* is considered capable to express and activate biosynthesis of such complex natural products (Wenzel et al. 2005; Gross et al. 2006). This has been made possible with the better understanding of metabolic

Table 8.7 List of natural products synthesized in *P. putida* strains by heterologous gene expression and strain engineering

Product	Producer org	<i>P. putida</i> strain	Expression strategy	References
Rhamnolipids				
Mono-RL	<i>P. aeruginosa</i>	KT2442	P_{tac} <i>rhlAB</i> , pl	Ochsner et al. (1995)
		KT2440	P_{tac} <i>rhlAB</i> , pl	Setoodeh et al. (2014)
Mono- and di-RL	<i>P. aeruginosa</i>	KT2440	<i>rhlAB/rhlABM</i> , pl	Cao et al. (2012)
	<i>B. glumae</i>	KT2440	P_{tac} <i>rhlAB' C'</i> , pl	Blank et al. (2013)
Terpenoids				
Zeaxanthin	<i>P. ananatis</i>	KT2440	$rhaP_{BAD}$, <i>crtEIBYZ</i> + isoprenoid gene <i>E coli</i>	Beuttler et al. (2011)
β -Carotene Zeaxanthin	<i>P. ananatis</i>	KT2440	P_{T7} , <i>crtEΔXYIBZ</i>	Loeschcke et al. (2013)
Polyketide/non-ribosomal peptides				
2,4-DAPG	<i>P. fluorescens</i>	KT2440	P_{native} / <i>PchrPpu</i> , <i>phlACBDE</i>	Martinez et al. (2004)
Myxochromide S	<i>S. aurantiaca</i>	KT2440	P_m , <i>mchABC</i> , chr	Wenzel et al. (2005)
Myxothiazol A	<i>S. aurantiaca</i>	KT2440	P_m , <i>mtaBCDEFG</i> , chr	Perlova et al. (2006)
β -Lactam	<i>L. lactamgenus</i>	IFO14164	P_{tac} , <i>pcbABCcefEFDbla</i> , pl	Kimura et al. (1996)
Syringolin A	<i>P. syringae</i>	P3	P_{native} , <i>sylABCDE</i> , cos	Remal et al. (2009)
Amino acid-derived compounds				
Phenol	<i>P. agglomerans</i>	S12	NagR/pNagAa, <i>tpl</i> , pl	Wierckx et al. (2005)
t-Cinnamate	<i>R. toruloides</i>	S12	P_{tac} , <i>pal</i> , pl + mutagenesis	Nijkamp et al. (2005)
<i>p</i> -Hydroxy-styrene	<i>R. toruloides</i>	S12	NagR/pNagAa, <i>pal pdc</i> , pl	Verhoef et al. (2009)
Phenazine PCA	<i>P. fluorescens</i>	WCS358r	P_{tac} , <i>phzABCDEFGF</i>	Glandorf et al. (2001)
Pyocyanin	<i>P. aeruginosa</i>	KT2440	NagR/pNagA, <i>phzA1B1C1D1E1F1G1</i> , <i>phzMS</i>	Schmitz et al. (2015)
<i>p</i> -OH Benzoate	<i>R. toruloides</i>	S12	P_{tac} , pl, Δ <i>gcd</i> , <i>xylAB_FGH</i>	Meijnen et al. (2011a, b)

P_{tac} , tac promoter; P_{native} , native promoter, genes expressed; pl, plasmid; cos, cosmid; chr, chromosome

pathways, enzymic analysis and genomic sequences of more and more organisms being available. Two strains of *P. putida* that have been particularly useful are KT2440 (Nelson et al. 2002) and S12 (Kueppler et al. 2015), and their genomic sequences along with many other *Pseudomonas* strains are available for comparison. Production of myxochromide S, a natural product from myxobacteria, has been successfully achieved in *P. putida* KT2440, and the yield was five times of that by parent strain, *Stigmatella aurantiaca* (Stephan et al. 2006). *P. putida* FG2005 has been used as a heterologous host for the production of myxothiazol (Gross et al. 2006). The techniques of metabolic engineering and genetic engineering using transposons allow efficient transfer of large gene clusters for heterologous production of complex natural products (Fu et al. 2008). Biosynthesis of carotenoids such as zeaxanthin has been achieved in *P. putida* KT2440 (Puchałka et al. 2008; Beuttler et al. 2011). This has laid the way for novel possibilities to use *P. putida* as chassis for incorporating different gene functions and a platform for production for high-value products. Currently *P. putida* has been used for the production of a number of products (Table 8.7) including rhamnolipids and their modification from mono-rhamnolipid to di-rhamnolipids, terpenoids, polyketides/non-ribosomal peptides and a number of aromatic and nonaromatic metabolites (Loeschcke and Thies 2015) (Table 8.7).

8.8 Environmental Applications of *Pseudomonas*

Pseudomonas have been successfully exploited for the bioremediation of environment contaminated with toxic chemicals such as organic solvents, petroleum hydrocarbons and alkaloids, recalcitrant man-made chemicals such as pesticides and herbicide and a variety of other organic chemicals (Arora et al. 2009, 2010). Important *Pseudomonas* species used for bio-augmentation of degradation of chemical pollutants and bioremediation are *P. putida* (organic solvents, etc.), *P. mendocina* (toluene), *P. pseudoalcaligenes* (cyanide), *P. veronii* (aromatic compounds), *P. knackmussii* and *P. stutzeri* (chlorinated hydrocarbons).

Polycyclic aromatic hydrocarbons (PAH) are hazardous in the environment due to their properties of being mutagenic and carcinogenic in nature. A number of pseudomonads such as *P. putida*, *P. fluorescens*, *P. paucimobilis*, *P. vesicularis*, *P. cepacia*, *P. testosteroni* and *P. aeruginosa* are capable of degrading a variety of PAH in the environment (de Lorenzo 2008). For details reader is referred to Chap. 9 on degradation and bioremediation of pollutants.

8.9 Agricultural Applications

8.9.1 Biocontrol of Phytopathogens

The tripartite ecosystem comprising of plant-soil-microorganism greatly contributes to plant growth. *Pseudomonas* spp. are the predominant bacteria that colonize plant root system and produce allelochemicals which suppress the growth of other microorganisms sharing the same habitat. This activity of pseudomonads has been exploited as biocontrol of plant diseases, and *P. fluorescens* strains are the best studied bacteria for biocontrol (O'Sullivan and O'Gara 1992; Kim et al. 2011). Their action is mediated through the production of *siderophores* which offer furious competition to other organisms in the rhizosphere for iron, particularly under iron-limiting conditions. Siderophores like pyoverdine produced by *Pseudomonas* sp. have very high affinity for iron and deprive pathogenic fungi of this essential nutrient. *Pseudomonas* are also capable of drawing iron from siderophores produced by other microorganisms in the rhizosphere (Loper and Henkels 1997, 1999; Whipps 2001). Siderophore synthesis is regulated by global regulators GacS and GacA; the sigma factors RpoS, PvdS and Fpv1; quorum-sensing autoinducers such as *N*-acetyl homoserine lactone; and site-specific recombinase (Ravel and Cornelis 2003).

8.9.1.1 Production of Antimicrobials

Production of antibiotics as a suppressive agent by *P. fluorescens* has received a lot of attention for the last couple of decades. Important among these are 2,4-diacetylphloroglucinol (DAPG), amphisin, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, tropolone, cyclic lipopeptides and hydrogen cyanide (Défago 1993; Raaijmakers et al. 2002; Nielsen et al. 2002; Nielsen and Sørensen 2003). Some of these are finding use as pharmaceuticals (Di Santo et al. 1998; Isnansetyo et al. 2003) while others are useful as biocontrol agents for plant diseases and plant growth promoters. Antibiotic production is closely linked to overall metabolic status of the cell. The regulatory cascades involve GacA/GacS or GrrA/GrrS, RpoD and RpoS, *N*-acylhomoserine lactone derivatives and positive autoregulation (Schnider-Keel et al. 2000; Bloemberg and Lugtenberg 2001).

8.9.1.2 Production of Lytic Enzymes

A number of microorganisms produce hydrolases acting on cell wall of fungi thus affecting the spore germination and growth of pathogenic fungi in the rhizosphere. *Pseudomonas stutzeri* produces extracellular chitinase and laminarinase and digest and lyse mycelia of *Fusarium solani*. *Pseudomonas cepacia* (*B. cepacia*) synthesizes β 1,3-glucanase and disrupts the integrity of cell walls of *Rhizoctonia solani*, *Sclerotium rolfsii* and *Pythium ultimum* (Fridlender et al. 1993).

Detoxification and degradation of virulence factors are important aspects of several biocontrol agents as they are able to detoxify toxins produced by pathogens, e.g. *Burkholderia cepacia* and *Ralstonia solanacearum* (both were earlier classified as *Pseudomonas*) hydrolyse fusaric acid, a phytotoxin produced by *Fusarium*

species. At the same time the pathogens produce toxins with broad spectrum of activity and can suppress growth of microbial competitors or detoxify antibiotics produced by biocontrol microorganisms as a self-defence mechanism against biocontrol agent (Toyoda et al. 1988; Compant et al. 2005). Endophytic bacteria also synthesize metabolites with antagonistic activity towards plant pathogens. *Pseudomonas fluorescens* strain FPT9601 can synthesize DAPG and deposit the DAPG crystals around and in the roots of tomato thus inhibiting the growth of pathogens. Apart from biocontrol activities, certain bacteria trigger 'induced synthetic resistance' (ISR) which is similar to 'systemic acquired resistance' (SAR), but ISR induces a hypersensitive reaction thus limiting the pathogen to local necrotic lesions of brown adicesed tissue (Van Loon et al. 1998). *P. fluorescens* EPI induces ISR against sugarcane red rot caused by *Colletotrichum orbiculare* and *P. fluorescens* 63-28 against *F. oxysporum* f. sp. *radicis-licopersici* of tomato and *P. ultimum* and *F. oxysporum* f. sp. *pisi* on pea roots and *P. denitrificans* 1-15 and *P. putida* 5-48 against *Ceratocystis fagacearum* on oak (Bloembergen and Lugtenberg 2001). Although *P. fluorescens* has been demonstrated as a successful biocontrol agent in various applications, it does not match the broad spectrum of chemical pesticides. In contrast *P. fluorescens* are ecofriendly, and their use as disease control measure is expanding and is being successfully used in cereal crops.

P. putida have been reported to detoxify and degrade herbicides such as glyphosate and atrazine which are used as non-selective weedicides. Transgenic plants resistant to herbicide have been engineered. Future application of siderophore from *Pseudomonas* has been proposed to control lactic acid fermentations. The non-oxenic fermentations could be directed towards lactic acid fermentation by complexation of iron through the addition of 2,2-dipyridyl.

Ice-nucleating bacteria, *Pseudomonas syringae*, cause a lot of damage to field crops as they possess a membrane protein enabling them for crystallization of supercooled water. Mutants lacking ice-nucleating genes (IN^{-}) are used for protection against frost damage by spraying these on the plant foliage and creating a competition between the two for space and nutrients. The wild type ones (IN^{+}) are being used for snow making and have potential in food production and texturing of frozen foods (Margaritis and Bassi 1991).

Pseudomonas fluorescens group (Mulet et al. 2010) harbouring rhizosphere and root surface colonizers is an important group from the point of view of plant growth promotion and plant protection. Particularly plant-beneficial species are *P. fluorescens*, *P. protegens* and *P. chlororaphis*. They protect the plants against important fungal diseases caused by *Gaeumannomyces* sp., *Thielaviopsis*, *Rhizoctonia*, *Fusarium oxysporum* and *Pythium* sp. *Pseudomonas fluorescens* group being excellent root colonizers effectively compete with fungal pathogens for rhizosphere niches and macro- and micronutrients (Mercado-Blanco and Bakker 2007; Lugtenberg and Kamilova 2009) by production of high-affinity iron chelators as well as a cocktail of secondary metabolites with potent antifungal activity.

Pseudomonads may use these compounds also for self-defence against predatory protozoa and nematodes (Bjørnlund et al. 2009). Pseudomonads producing 2,4-diacetylphloroglucinol (DAPG), phenazines or cyclic lipopeptides are naturally

suppressive to plant diseases such as take-all of wheat, black root of tobacco and *Rhizoctonia* of sugar beet. Besides direct antagonistic effect, several root-colonizing pseudomonads act indirectly by activating the plant defence mechanism such as ISR (induced systemic resistance) (van de Mortel et al. 2012; Zamioudis and Pieterse 2012; Balmer et al. 2013).

Thus *Pseudomonas* sp. can be exploited for plant growth promotion and health using two strategies. The first envisages that suitable cropping system be adapted by modifying tillage, iron rotation, intercropping and soil amendments with composting and green manuring. The second strategy is the use *Pseudomonas*-based biopesticides as seed treatment, soil drench and foliar spray. Several products based on plant-beneficial pseudomonads have been commercialized in US markets, e.g. AtEze (*P. chlororaphis*) against *Pythium*, *Rhizoctonia* and *Fusarium* root diseases of vegetables and ornamentals in green houses; Blightban A506 (*P. fluorescens*) against blight of apple and peach; Bio-save 10LP/11LP (*P. syringae*) for control of postharvest diseases of fruits and potato (Fravel 2005). *P. chlororaphis* formulations Cedamon and Cerall are used in Europe as seed treatments of cereals for control of seed-borne diseases (Mark et al. 2006). Proradix, a *Pseudomonas*-based product is marketed for treatment of potato tubers for control of *Rhizoctonia*, *Phytophthora*, *Streptomyces* and *Erwinia* (Buddrus-Schiemann et al. 2010).

8.9.2 Insecticidal Activity of *Pseudomonas fluorescens*

Some strains of *P. fluorescens* exhibit insecticidal activity against agricultural pest insects such as aphids, beetles and termites (Hashimoto 2002; Otsu et al. 2004; Devi and Kothamasi 2009). A number of *P. fluorescens* strains are capable of killing fruit fly *Drosophila melanogaster* (Olcott et al. 2010). Molecular basis of insecticidal activity include proteins or metabolites such as HCN and lipopeptide-viscosin and orfamide (Jang et al. 2013). Genome sequence analysis of *P. fluorescens* CHAO (now *P. protegens* CHAO) has revealed that the genome possesses a gene coding for protein that is similar to insect toxin Mcf1 of the entomopathogen *P. luminescens* (Pechy-Tarr et al. 2008). In contrast to gene *mef1*, the *P. fluorescens* strains Pf-5 and CHAO possess a gene *fit* that codes for insect toxin, fit. The fit toxin gene has also been identified in *P. protegens* and *P. chlororaphis* (Loper et al. 2012; Ruffner et al. 2009).

The microbial control agents being environmentally friendly and harmless to human beings and mammals hold great potential as components of IPM systems.

8.10 Conclusion

Expanding databases and techniques of system biology hold great potential for biotechnological exploitation of *Pseudomonas*. Recent example of recombining large segment, iJN746, representing 746 genes, 950 reactions and 911 metabolites

involving biotechnologically relevant pathways including pathways of polyhydroxyalkanoates synthesis and catabolic pathways of aromatic compounds is a step in this direction. *Pseudomonas* offers a robust system and suitable platform for further exploring its potential for commercial biotechnology and development of novel products with commercial value. Cost-effectiveness of any industrial process is important for its success. Low-cost renewable feedstock such as lignocellulosics need to be harnessed and coupled to commercial processes using *Pseudomonas*.

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Biodegradation and Bioremediation of Organic Chemical Pollutants by *Pseudomonas*

9

Rachhpal S. Kahlon

Contents

9.1	Introduction	344
9.2	Important Organic Chemical Pollutants	347
9.2.1	Petroleum Hydrocarbons	347
9.2.2	Chlorinated Organic Compounds	350
9.2.3	Pesticides	351
9.3	<i>Pseudomonas</i> as Degradative Bacteria	352
9.4	Biodegradation of Pollutants by <i>Pseudomonas</i>	355
9.4.1	Chlorinated Aliphatics	357
9.4.2	Aromatic Compounds: BTEX	357
9.4.3	Degradation of Polycyclic Aromatic Hydrocarbons	361
9.4.4	Degradation of Chlorinated Aromatic Compounds	366
9.4.5	Genetic Basis of Degradative Pathways in <i>Pseudomonas</i> sp.	375
9.5	Degradation of Pesticides	382
9.5.1	Organochlorinated Insecticides	384
9.5.2	Organophosphates	387
9.5.3	Carbamates	388
9.5.4	Atrazine	389
9.5.5	Enzyme and Genes in Pesticide Degradation	390
9.6	Bioremediation	392
9.6.1	Types of Bioremediation	392
9.6.2	In Situ Bioremediation	394
9.6.3	Ex Situ Bioremediation	396
9.6.4	Bioaugmentation	397
9.6.5	Rhizoremediation	400
9.6.6	Factors Affecting Bioremediation	401
9.7	Biotechnology and Emerging Technologies	402
	References	404

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Abstract

Over exploitation of natural resources and indiscriminate use of hazardous chemical substances has resulted in environmental pollution and ecological imbalance, leading to climate change and global warming. Efforts have been afoot to develop an eco-friendly, low-cost, and easy-to-use technology system to accomplish complete degradation (mineralization), partial degradation resulting in smaller molecules that are nontoxic or less toxic, or biotransformation or reduction of highly electrophilic groups to less toxic compounds. Members of the genus *Pseudomonas* which are endowed with a vast catabolic versatility and genetic diversity were the natural choice and have been exploited for techniques of in situ and ex situ bioremediation. Particular attention was paid to persistent organic pollutants such as polychlorinated phenols and biphenyls, polycyclic aromatic hydrocarbons, petroleum hydrocarbons, and pesticides. Plasmids encoding catabolic pathways have been used to expand the metabolic potential of *Pseudomonas* for use in sites contaminated with multiple pollutants. New techniques of molecular biology and genetic engineering have been used to engineer efficient and self-limiting strains. The expansion of knowledge of genomics, proteomics, transcriptomics, and metabolomics and bio-information databases have proved useful to predict the metabolic potential of the organism in contaminated environments. Simultaneously, emphasis is also on improving the efficiency of natural microflora for bioremediation with minimum disturbance at the site. The technique of rhizoremediation by using root-colonizing bacteria with degradative capabilities such as *Pseudomonas putida* KT2440 and *P. fluorescens* has been found useful for degradation of pollutants in the root zone and can even travel to deeper layers of soil. The technique holds great potential for restoration of polluted sites.

9.1 Introduction

The last century witnessed a phase of large-scale industrialization, development, and population growth, resulting in unprecedented exploitation of natural resources and disturbances in the elemental cycle. This has led to global climate change, and its impact is being felt by way of increase in earth's surface temperature, increased drought or flooding amplitude, loss of soil carbon content, etc. This has been further complicated by the use of manmade hazardous (xenobiotic) chemicals which have accumulated in the environment. All this has put the entire biological system including human beings under stress and the sustainability of agriculture is threatened. Consequently, there is growing concern about the climate change and overall degradation of the environment. Much of this is arising from growing production and use of fossil fuels, extensive use of insecticides, herbicides, fungicides, etc. in agriculture, and use of chemicals such as solvents, dyes, plasticizers, etc. in the industry. Oil spills due to releases of crude oil from tankers,

offshore platforms, drilling rigs, and oil wells as well as spillage of the finished products (gasoline, diesel, solvents, etc.) cause considerable damage to the natural ecosystem. Oil spills have damaged the natural ecosystems in Alaska, the Gulf of Mexico, Galapagos Islands of France, and Niger Delta region of Nigeria, etc. Pollution of the environment by petroleum hydrocarbons, halocarbons, nitroaromatics, polychlorinated biphenyls (PCBs) solvents and extensive use of pesticides in agriculture and public health has had an enormous effect on the environment and ecological balance of species. Oil penetrates into the structure of plumage of birds and fur of mammals, thereby reducing their insulating ability and thus making them vulnerable to fluctuating temperatures, extinction of species due to feeding of insects, and grains contaminated with pesticides. The increased surface runoff could lead to contamination of water bodies by pesticides and heavy metal ions, thereby affecting the aquatic biota. Thus the environmental degradation coupled with climate change is a challenge for the society to cope up with. However, the effects of the climate change may be slow, variable, and difficult to detect, and many species may adapt to this.

In the soil ecosystem, soil acts as a big sink and must be taken care of while the intensive and unsustainable agricultural practices contribute to the deteriorating soil health and loss of diversity (Jeffery et al. 2010). The ability of soil to recover from contamination largely depends upon the availability of water as a solvent and abundance and diversity of microbial population with ability to degrade contaminant (Bara Caracciolo et al. 2013). Thus the challenge before the scientific community is to tackle the problem of environmental degradation and restoration of soil health in a manner which is environmentally and economically sound. The answer to this is the technique of bioremediation, and it has been a subject of intensive studies for the last two to three decades.

Initially the release of pollutants, resulting from various human activities, into the environment went unnoticed, as the proposition of “microbial infallibility” got wide acceptability. Because of the ubiquitous presence of microorganisms and their vast metabolic potential it was considered that any molecule released into the biosphere will be taken care of by microorganisms and mineralized in the cycle of matter.

Today the waste products range from raw sewage to nuclear waste. Earlier such wastes were deposited by dumping into a deep hole dug in the soil. But this method is insufficient with the increase in the amount and variety of waste. Even the toxic material from such sites has begun to leak and pollute the underground water reservoirs. The alternative to this is the present-day technique “bioremediation,” i.e., the transformation or degradation of contaminants into nonhazardous or less hazardous chemicals as end products. Generally bacteria are used as agents of bioremediation though fungi, algae, and plants have also proved useful under certain conditions. The term bioremediation was first used in 1987 although the technique has been in use as far back as 600 BC as compost piles and the first sewage treatment plant was created in Sussex, UK, in 1891.

Microorganisms degrade organic chemical pollutants by three types of biochemical reactions:

1. Biotransformation in which the contaminating molecule is transformed into a less complex molecule by addition or removal of one or more reactive groups. The new molecule may be less toxic or more toxic. The process does not provide free energy thus does not support growth.
2. Biodegradation, i.e., the anaerobic microorganisms break down organic molecules yielding smaller molecules such as organic acids, aldehydes, methane, etc. in the absence of oxygen. These reactions support limited growth, as the energy yield is less than mineralization.
3. Mineralization is the process of complete oxidation of organic compounds by aerobic microorganisms into inorganic constituents, CO_2 , and H_2O and biomass accumulation. Some compounds also undergo cometabolism, i.e., the secondary substrate undergoes degradation when a microorganism is growing on another (primary) substrate as a source of carbon and energy (Diez 2010).

Bioremediation depends on the presence of appropriate microorganisms in suitable concentration, combinations, and environmental conditions. The intrinsic microflora of the contaminated environment is usually well adapted to the contaminating substance, as well as conditions of pH, temperature, moisture, and oxidation-reduction potential of the site. Degradation of organic chemical pollutants in soil and water, particularly under controlled conditions, got an impetus with the identification of degradative plasmids in *Pseudomonas* species for catabolism of a variety of compounds such as camphor, salicylate, naphthalene, chlorinated benzoic acids, xylene, toluene, nicotine, etc. (Chakrabarty 1987). During the same periods, other organisms degrading 2,4-D and related compounds were identified, thus laying the foundation of modern technique of bioremediation for complete degradation and transformation of contaminants rendering them nontoxic or less toxic in the environment. The vast metabolic potential, ability to grow on a simple medium, and presence in a variety of environmental niches make *Pseudomonas* the organism of choice for bioremediation. The present chapter will exclusively deal with the role of *Pseudomonas* in degradation of organic pollutants and bioremediation of a contaminated environment.

Biotechnological processes for pollution control are advantageous over the physical and chemical processes as a successful biological process can convert the organic pollutant to inorganic end product in the form of CO_2 and water. Besides, these are low cost and offer an in situ treatment. However, the biological treatment processes suffer from limitations such as susceptibility to toxic effects and are operative at a narrow range of physiological conditions of the biological system. Biological agents are also more difficult to model and control as compared to physicochemical processes (Mukherjee 2014). The challenging problems for biological approaches are:

1. The microbial enzymes are highly specific and no single organism or community can effectively destroy all the organic wastes as a large number of chemicals may be present in the waste.

2. The concentration of the pollutant may be low in oligotrophic range and may not be enough to support growth and energy requirements of the organism.
3. The contaminated sites and industrial waste containing the target chemical may also contain other toxic chemicals which may be biochemically incompatible and prove toxic to the degrading organism.
4. Bioavailability of the chemical in nature is a serious problem. The nonpolar compounds rapidly adsorb onto particulate matter in soil, sediment, and water thus becoming less bioavailable to the microbial systems.

9.2 Important Organic Chemical Pollutants

The list of the pollutants is ever-growing and includes all sorts of chemicals falling in the categories petroleum hydrocarbons including both aliphatic and aromatic compounds and their substitutes as halogenated, nitro-aromatic compounds, and phthalate esters. Presently, it is estimated that over 100,000 chemical substances find use in the industry and many of these have properties similar to persistent organic pollutants. Some of these are used in the industry as solvents and for chemical synthesis, while some find wide application in agriculture as fertilizers, pesticides, herbicides, etc. (Fig. 9.1).

Compounds like polycyclic hydrocarbons (PAHs), dibenzo-*p*-dioxins, and dibenzofurans are released during the combustion processes. The concentration of the contaminant at the particular sight depends on the amount present, rate of release of the compound into the environment, stability of the compound, and its biological or nonbiological degradation. Important organic pollutants (Table 9.1) are classed as under.

9.2.1 Petroleum Hydrocarbons

Petroleum hydrocarbons are naturally present and are extracted in the form of crude oil and transported worldwide where it is subjected to refinement and separation into different fractions which find a variety of uses in the industry as fuels or gasoline, lubricants, etc. During these processes the crude oil can be released into the environment as a result of accidents or inevitable spillage leading to serious pollution problems (Thouand et al. 1999) and disturbances to both biotic and abiotic compounds of the ecosystems. Some of the hydrocarbon compounds are known to be mutagenic, carcinogenic, and neurotoxic in nature. Aromatic hydrocarbons, e.g., benzene, toluene, ethyl benzene, and xylene (BTEX) and naphthalene and related petrochemicals are extensively used as fuels and industrial solvents. Phenols and their derivatives are released into the environment as waste products from the industry and degradation of plant cell biomass, particularly lignin.

Crude oil is heterogeneous and several hundred hydrocarbon compounds can be separated from it. These comprise of two elements, hydrogen and carbon in the ratio of 2:1. Elements such as nitrogen, sulfur and oxygen may be present and all of these

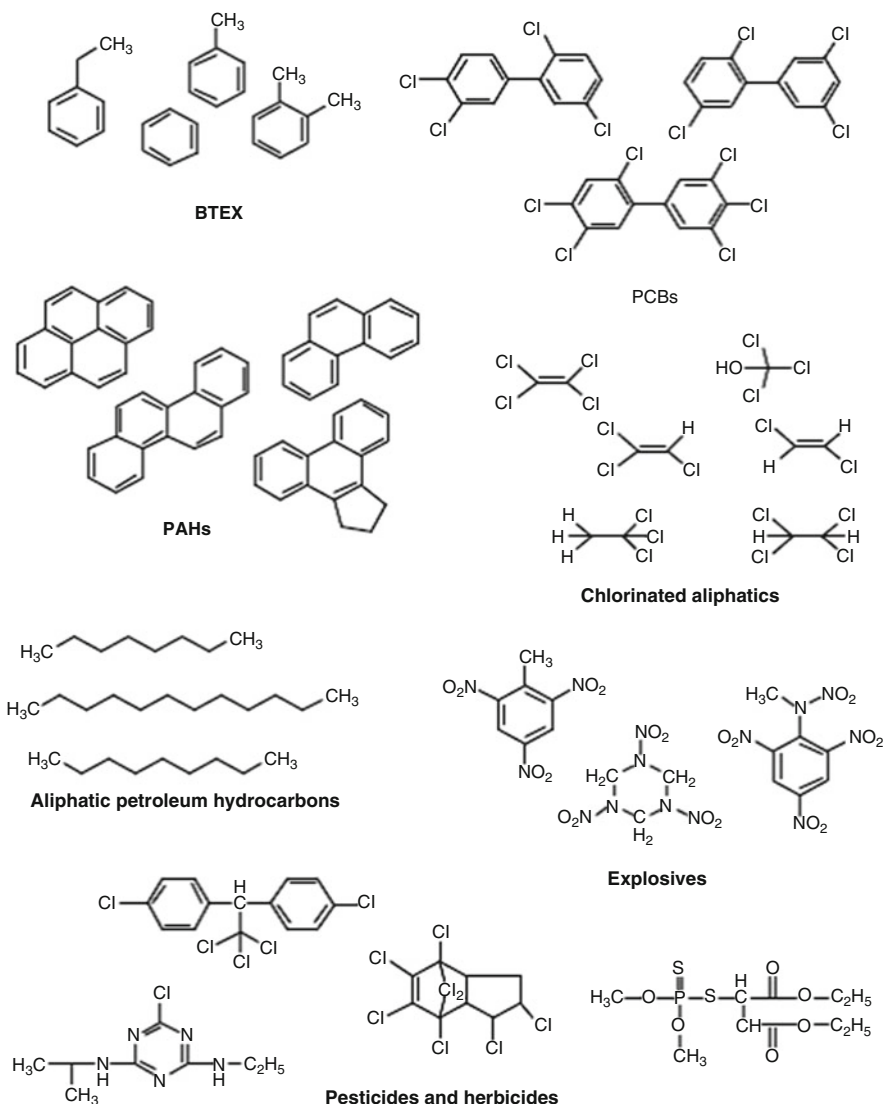


Fig. 9.1 Common organic chemical pollutants

constitute less than 3 % (v/v). Trace elements like phosphorus and heavy metals constitute less than 1 % (v/v). On a structural basis, the hydrocarbons in crude oil are classified as alkanes (normal or iso), cycloalkanes, and aromatics. Alkenes are unsaturated analogs of alkanes which are mostly formed during cracking process of refining. Hydrocarbons comprising of C_6 – C_{10} in the form of alkanes or alkenes or aromatics form the gasoline component used as fuel. Hydrocarbons, C_{11} – C_{28} form

Table 9.1 Important organic chemical pollutants and their source in the environment

Type	Example	Source
<i>I. Petroleum hydrocarbons</i>		
Crude oil	Crude oil and sludge	Spillage from tankers, leakage, etc.
Aromatics	BTEX, phenols, biphenyls	Oil production and storage Airports, seaports, paint and chemical industry, etc.
Polycyclic aromatic hydrocarbons (PAH)	Naphthalene, anthracene, styrene, fluorene, pyrene, benzo(a)pyrene	Lignin from biomass, oil production and storage, coke plants, landfills, etc.
<i>II. Chlorinated compounds</i>		
Alkanes/Alkenes	Chloromethane, carbon tetrachloride, tetrachloroethane di-, tri-, and tetrachloroethylene	Chemical industry Dry cleaners Electrical manufacturing
Aromatics	Chlorobenzenes and derivatives, pentachlorophenol, 4-chlorophenol	Timber treatment Land fills
Chlorobenzoates	2-, 3-, 4-chlorobenzoates 3,5-dichlorobenzoates	Chemical manufacturing degradation products of chlorobiphenyl and DDT
PCBs	4-chlorobiphenyl Dichlorobiphenyl	Electrical manufacturing Power stations
Dioxins	Polychlorinated-dibenzo- <i>p</i> -dioxins (PCDD) TCDD, etc.	By-product of incineration and burning
Trinitrotoluene	2,4,5-trinitrotoluene	Explosives
<i>III. Pesticides</i>		
Organochlorinated	DDT, HCH, lindane, aldrin, dieldrin, chlorodane	Agriculture use, industry and dumps, timber treatment
Organophosphates	Malathion, parathion, methyl parathion, diazinon	-do-
Carbamates	Sevin, carbaryl	-do-
Pyrethrin	Pyrethrins and pyrethroids	-do-
<i>IV Herbicides</i>		
Phenoxy herbicides	2,4-D; 2,4,5-T ^a	Agriculture
Triazines	Atrazine	Agriculture
Urea herbicides	Dichloralurea, isonoruron, isouron, etc.	Agriculture

^a2,4,5-T is highly toxic not used in agriculture

the diesel fraction mostly used in automobile and C₂₈–C₃₅ are the lubricants. Aromatics are either the C₆H₆ structures with different substituents or the aromatic cyclic structures which may be fused together to form polycyclic aromatic hydrocarbons, e.g., naphthalene, styrene, etc. Another fraction are asphaltenes comprising of phenols, fatty acids, ketones, esters, and porphyrins. Resins are structures having N, O, or sulfur atoms such as pyridines, quinolines, carbazoles,

sulfoxides, and amides. A partially oxygenated, highly condensed asphaltic fraction also occurs in crude but not in refined petroleum (Atlas and Bartha 1973; Bartha 1986). Alkanes in the range of C_{20} – C_{40} are referred as “waxes” which are hydrophobic solids at the physiological temperatures.

Polycyclic aromatic hydrocarbon (PAH) concentration in soil varies between $1\text{ }\mu\text{g}$ and 300 g kg^{-1} soil depending on the contamination source like combustion of fossil fuel, gasification, and liquefaction of coal, incineration of waste, etc. (Bamforth and Singleton 2005). Incomplete combustion of organic substances gives out about 100 different PAHs as pollutants. Few of these are used in medicine, dyes, plastics, and pesticides. Others have no industrial use and six of these are listed among 126 priority pollutants by EPA, USA (1998).

PAHs are ubiquitous organic pollutants formed by fusion of two or more benzene rings. PAHs are found in the environment after the disposal of coal processing wastes, petroleum sludge, asphalt, creosote, and other wood preservative waste. These can persist long because of their low solubility and also pose health hazards because of their mutagenic, toxic, and carcinogenic properties (Patnaik 1992).

9.2.2 Chlorinated Organic Compounds

Chlorinated organic compounds are an important group of pollutants after petroleum hydrocarbons. Halogens constitute the 7th main group within the periodic system of elements and among these chlorine is the most abundant. Chemically halogens form very stable bonds with carbon and are strongly electronegative. Chlorinated compounds are highly persistent in the environment and can enter the food chain as they show poor water solubility and high fat solubility. Organochlorine compounds find wide application in the industry as organic solvents as well as in agriculture because organochlorine compounds are used as pesticides, herbicides, etc.

Naturally in the biosphere approximately 1500 different types of halogenated substances are produced by plants, animals, and microorganisms. In some cases the concentrations of these compounds exceed anthropogenic level.

Chlorinated aliphatics are short-chain chlorinated aliphatic compounds such as chloromethane, dichloromethane, chloroform, carbon tetrachloride (TETRA), dichloroethane (DCE), trichloroethane (TCE), and tetrachloroethane (TeCE) which are mainly used as solvents in dry cleaning, as degreasers, and in manufacturing of electronic components and pesticides. Because of their widespread application, these are common contaminants of soil and water. The long chain chloroalkanes and paraffins are used as plasticizers in paints, rubbers, and plastic materials. Solvents, like TCE and carbon tetrachloride, cause pollution because of their large-scale production and industrial use as solvents.

Haloaromatics particularly chlorobenzenes are highly volatile, lipophilic and toxic and subject to bioaccumulation. Other chloroaromatics include dichloroanilines and chloromethyl anilines used for the manufacture of herbicides, dyes,

pigments, and pharmaceuticals. Chloronitrobenzenes are used as starting material for synthesis of dyes, pesticides, and rubber chemicals.

Chlorophenols and pentachlorophenols (PCP) are used as wood and leather preservatives, insecticides, and herbicides. Chlorinated derivatives of benzoic acids are water soluble and show low toxicity and are degradable in natural environment. They are formed as degradation products of polychlorinated biphenyls and herbicides; e.g., 2,4-dichlorophenoxy acetic acid is widely used as herbicide and 2,4-dichlorophenol is formed as its cleavage product. Polychlorinated biphenyls are used as insulating liquids for transformers, grease for vacuum pumps, and turbines and coolants. They have low toxicity and high chemical stability.

PCBs used as hydraulic fluids, plasticizers, adhesives, fire retardants, and dielectric fluids in transformers are toxic, carcinogenic, and persistent. Polychlorinated dibenzodioxins and dibenzofurans are recalcitrant and their chlorinated derivatives are carcinogenic (Kaiser 2000).

9.2.3 Pesticides

Organochlorinated pesticides showing broad and extensive spectrum of activity against insects were introduced in the middle of last century. Among these, DDT, aldrin, dieldrin, and benzene hexachloride (BHC; now referred as hexachlorocyclohexane, HCH) found widespread application for insect pest control in agriculture and in public health as a measure to control insect vectors for vector-borne diseases such as malaria. Although these were very effective as insecticides, they proved to be highly recalcitrant with half-life extending to several years. Thus their residues were detected in soil, water, and a variety of foods including vegetables, grains, fish, meat, and even milk and milk products. So much so that human milk was detected to contain high levels of DDT as molecules are fat soluble and enter the food chain after they are taken up by crop plants. The use of most of these has been banned or restricted to certain areas of operation such as malaria control. Organochlorine compounds accumulate in fatty tissue of animals and enter the food chain due to their prolonged persistence. HCH comprises of five isomers: α (60–70 %), β (5–12 %), γ (10–12 %), δ (6–10 %), and ϵ (3–4 %). Gamma isomer (lindane) is less stable and biodegradable and is allowed for agricultural use, while all other isomers which are more stable and problematic have been banned. The lindane-contaminated sites have been identified and are being reclaimed by using microorganisms (Willett et al. 1998).

2,4-dichlorophenoxy acetic acid (2,4-D) is a commonly used herbicide and is known to be easily degraded in soil. Its isomer, 2,4,5-T (orange compound) shows very high herbicidal activity but is highly stable and toxic; thus its use is not permitted. Other chlorinated compounds widely used in agriculture are polychlorinated biphenyls, pentachlorophenols, and chlorobenzoates. Chlorobenzoates are important intermediates in the degradation of DDT, chlorinated biphenyls, and chlorophenols leading to formation of chlorocatechols.

Organophosphate pesticides act on insects by causing nerve toxicity by targeting enzyme acetylcholinesterase. Commonly used organophosphates are parathion, malathion, methyl parathion, chlorpyrifos, diazinon, fenitrothion, etc. These are easily degraded by hydrolysis on exposure to sunlight, air, and soil; however, small amounts have been detected in food and drinking water. Thus they pose risk because of their high toxicity. Parathion has been designated as most dangerous and carcinogenic and its use as a pesticide has been banned. Prolonged and repeated exposure can lead to chronic nerve disorders while poisoning by high doses is lethal (Shah and Devkota 2009).

Carbamates are organic compounds derived from carbamic acid (NH_2COOH). The functional groups are carbamic acid or carbamate esters (e.g., ethyl carbamate). Carbamate ester-based insecticides are aldicarb, carbofuran, carbaryl (Sevin), etc. Like organophosphates they also act by inactivating the acetylcholinesterase, but the action is reversible, whereas the organic phosphates cause irreversible inhibition of acetylcholinesterase, a more severe form of nerve toxicity. Carbamates also have low persistence in the environment.

Pyrethrins and pyrethroids are a group of botanical insecticides derived from flowers of *Chrysanthemum cinerariaefolium* and are considered safe substitutes for organophosphates and carbamates which show acute toxicity toward birds and mammals. The active ingredient of pyrethrin has six components, pyrethrin 1, pyrethrin 2, cinerin 1, cinerin 2, jasmolin 1, and jasmolin 2 which show insecticidal activity. Pyrethroids are chemically synthesized insecticides based on pyrethrins and have been suitably modified to enhance their stability in sunlight. A large number of pyrethroid insecticides are available for use in agriculture, public health, and as well as for household insect pests. Important among these are permethrin, cypermethrin, deltamethrin, allethrin, fenpropathrin, etc. They are toxic to insects at low doses and are fast acting. They show low toxicity toward mammals and birds but have high toxicity toward fish but are poorly soluble in water. Some pyrethroids may persist in the environment for a few days or weeks particularly if protected from sunlight while others such as allethrin break down within few minutes or hours after use.

Low toxicity of pyrethroids is attributed to (1) limited absorption of pyrethroids and (2) rapid biodegradation by liver enzymes. Insects lacking liver enzyme functions are highly susceptible (Reigart et al. 1999). Pyrethroids interfere with ionic conductance of the nerve membranes by prolonging the sodium current. Thus these are also nerve poisons active on axons in the peripheral and central nervous system by interacting with sodium channels in mammals and insects.

9.3 *Pseudomonas* as Degradative Bacteria

Pseudomonas are ubiquitously present and have been isolated from variety of environmental niches enriched with particular type of organic molecules being available. Among aerobic bacteria pseudomonads are most frequently isolated from the contaminated environments. During the last 50 years the concentration

Table 9.2 Important species of *Pseudomonas* and other aerobic bacteria degrading major persistent organic pollutants

<i>Pseudomonas</i>	<i>P. aeruginosa</i> , <i>P. canvexa</i> , <i>P. putida</i> , <i>P. fluorescens</i> <i>P. mendocina</i> , <i>P. paucimobilis</i> , <i>P. maltophilia</i> , <i>P. diminuta</i> , <i>P. pseudomallei</i> , <i>P. knackmussi</i> <i>P. montelli</i> , <i>Pseudomonas</i> spp., <i>P. chlororaphis</i> <i>P. azalacia</i> , <i>P. stutzeri</i>
<i>Burkholderia</i>	<i>B. cepacia</i> (<i>P. cepacia</i>), <i>B. tropicalis</i> , <i>Burkholderia</i> sp.
<i>Ralstonia</i>	<i>R. pickettii</i> (<i>P. pickettii</i>), <i>R. solanacearum</i> , <i>Ralstonia</i> sp.
<i>Comamonas</i>	<i>C. testosteroni</i> (<i>P. testosteroni</i>)
<i>Delftia</i>	<i>D. acidovorans</i>
<i>Sphingobium</i> <i>Sphingomonas</i>	<i>S. indicum</i> , <i>S. japonicum</i> , <i>S. francense</i> , <i>S. chlorophenolicum</i> , <i>Sphingomonas</i> sp.
Other genera	<i>Alcaligenes eutrophus</i> (<i>Cupriavidus necator</i> JMB134) <i>A. denitrificans</i> , <i>A. paradoxa</i> , <i>Arthrobacter</i> sp. <i>Acinetobacter</i> sp., <i>Enterobacter aerogenes</i> <i>Agrobacterium</i> sp., <i>Micrococcus</i> sp., <i>Rhodococcus</i> sp. <i>Hydrobacterium</i> sp., <i>Erwinia</i> sp., <i>Xanthomonas</i> sp.

of pollutants such as polycyclic aromatic hydrocarbons (PAHs), BTEX (benzene, toluene, ethyl benzene, and xylene), chlorophenols, nitrophenols, polychlorinated biphenyls, organic solvents, and organochlorinated pesticides has been increasing. Contamination of soil, wastewater, and aquifers by these results from coal gasification, oil refineries, petrochemical plants and spills, and industries involved in synthesis of industrial chemicals, herbicides, and pesticides. Exploitation of the degradative potential of *Pseudomonas* got recognition and boost with the characterization of plasmids involved in the degradation of number of hydrocarbons and compounds in Prof. Gunsalus's Laboratory in the 1970s followed by the patenting of live organism *Pseudomonas* (super bug) capable of degrading a number of hydrocarbons for growth (Dunn and Gunsalus 1973; Chakrabarty 1981; Jimenez et al. 2002). Important species of *Pseudomonas* and other bacteria degrading persistent organic pollutants are listed in Table 9.2.

Members of genus *Pseudomonas* have been isolated from sites contaminated with aliphatic and aromatic hydrocarbons and their substituents and utilize these as sources of carbon and energy. *Pseudomonas oleovorans* monooxygenase, the key enzyme, is a multiple polypeptide enzyme which contains an active site with two Fe atoms and bring about hydroxylation of *n*-alkane by transferring electrons from NADH to the monooxygenase (Lange and Que 1998) to form alkanol which is further metabolized by alcohol dehydrogenase, aldehyde dehydrogenase, acetyl-CoA synthase, and β -oxidation reaction forming acetyl-CoA that enters the TCA cycle in central metabolic pathway.

Important criteria that make *Pseudomonas* so versatile in metabolism and suitable for bioremediation include:

1. Broad specificity of enzymes involved in primary attack on xenobiotics makes them capable of degrading structurally analogous compounds. Toluene

dioxygenase of *P. putida* F₁ is capable of oxidizing trichloroethylene, *p*-chlorobenzene, phenol, 2,5-dichlorophenol, indol, indan, and indene (Golovleva et al. 1992). Similarly, dehalogenases of *Pseudomonas* sp. CBS3 and *P. pickettii* are able to dehalogenase, 2-, 4-, 2,4 chlorobenzoate and 4 bromo, 4-iodo-, 4-chloro, and 3,5-dichlorobenzoate (Thiele et al. 1997; Kocher and Kahlon 1999; Banta and Kahlon 2007).

2. Dioxygenases attack the thermodynamically highly stable compounds and incorporate two atoms of oxygen forming catechol and protocatechuate from a number of compounds.
3. Another feature of pseudomonads is simultaneous functioning of several enzymes involved in ring cleavage which is the rate-limiting step in degradation of xenobiotics, e.g., in *P. aeruginosa* catechol 2,3-dioxygenase, catechol 1,2-dioxygenase, and protocatechuate 3,4-dioxygenase are simultaneously active (Golovleva et al. 1992). Chloromuconate cycloisomerase from *Pseudomonas* sp. B13 and *Pseudomonas* sp. P51 have different substrate specificities. Thus, the multiple forms of enzymes have been useful in expanding their metabolic capacity of *Pseudomonas* strains (Loh and Cao 2008).
4. Genes coding for a number of catabolic peripheral pathways are borne on plasmids referred as degradative (D) plasmids. Plasmid-borne genes provide for flexibility and spread fast among populations by horizontal gene transfer (Chakrabarty 1981; Singh and Kahlon 1989).
5. Chemotaxis toward pollutants by way of transport system protein is another important feature of *Pseudomonas* and other biodegrading bacteria. The 4-hydroxybenzoate transporter protein PcaK of *P. putida* also acts as a major chemotaxis molecule for 4-hydroxybenzoate. A plasmid-coded membrane protein NahY of *P. putida* is also involved in chemotaxis of naphthalene (Grim and Harwood 1999). This mechanism may be an important parameter for degradation of pollutants in natural environment.
6. By another mechanism, the *Pseudomonas* capable of degrading organic solvents develop tolerance to solvents by altering the composition of their inner and outer membrane proteins. Two mechanisms such as increased biosynthesis of phospholipids in the outer membrane in *P. putida* JH-2000 and presence of solvent efflux systems in *P. putida* KT2442 have been reported to facilitate solvent tolerance and degradation (Kobayashi et al. 2000).

All these factors help establishment of *Pseudomonas* in different niches and scavenge environmental pollutants. Besides true *Pseudomonas* some of the genera now classified as *Pseudomonas* (*lato*) play an important role in degradation of chemical pollutants. Prominent among these are *Comamonas testosteroni*, *Burkholderia cepacia*, *Burkholderia* sp. FDS-1, *Arthrobacter chlorophenolicus*, *Sphingomonas* spp., *Sphingobium* spp., *Ralstonia* spp., *Cupriavidus necator*, *Acinetobacter* sp., etc.

9.4 Biodegradation of Pollutants by *Pseudomonas*

Bioremediation depends on the metabolic potential of microorganisms to detoxify or transform the pollutant molecule. For its degradation the substrate must be accessible and bioavailable to the degrading microorganisms (Antizar-Ladislao 2010). In soil the pollutant binds to the mineral and organic matter and for its bioavailability to soil microflora it must undergo desorption (release) from the soil particle. Another school of thought is that the organic pollutant compound is attacked by microorganism in the bound state to the soil particle and extracellular enzymes are particularly important in the process. The organic pollutants vary greatly with respect to solubility in water, volatility, and reactivity and thus affect the bioavailability in soil and water. The compound available in the form of solution in pore water is considered bioavailable as compared to the bound form which does not exert direct biological effect. Variations in bioavailability also exist because of the nature of pollutant, soil type, water content, and temperature.

Surface Active Agents as Enhancers of Bioactivity Biodegradation and bioremediation is not limited only by the toxicity and calcitrant nature of the molecule. But limited bioavailability, hydrophobic nature, and poor solubility play an important role in degradation of xenobiotics. Chemical surfactants such as Triton X-100, Tween-80, and sodium dodecyl sulfate result in increased desorption rates for sorbed pollutant from soil particles. The solubilization of hydrophobic contaminants is attributed to incorporation of the molecule into the hydrophobic core of micelles in solution. The mechanism involves lowering of interfacial tension, surfactant solubilization of hydrophobic organic compounds, and phase transfer of organic compounds from soil-sorbed to pseudo-aqueous phase (Laha et al. 2009). Surfactants have been reported to enhance mobilization and biodegradation of PAHs like naphthalene and phenanthrene. Likewise Triton and Brij 35 enhance solubility of DDT. Indeed the toxic effect of surfactants toward soil microflora prevents biodegradation of pollutants. Biosurfactants being biocompatible have certain advantages over synthetic surfactants, particularly for environmental applications. They also show high surface activity, stability toward heat and pH, and low toxicity, and above all they are biodegradable. However, the high cost of production restricts their use on a large scale.

Microbial Communities and Biodegradation In the natural environment there are diverse sources and chemically complex molecules forming pollution and perhaps more diverse are the microbial populations having vast metabolic capabilities to degrade pollutants (Ramakrishnan et al. 2011). The microorganisms coexist as communities and share strong interactions in the microenvironment and may be synergistic or antagonistic in nature. The potential to degrade organic pollutants varies among microbial groups as well as with concentration of the pollutant. Organic compounds such as toluene are toxic to microorganisms as

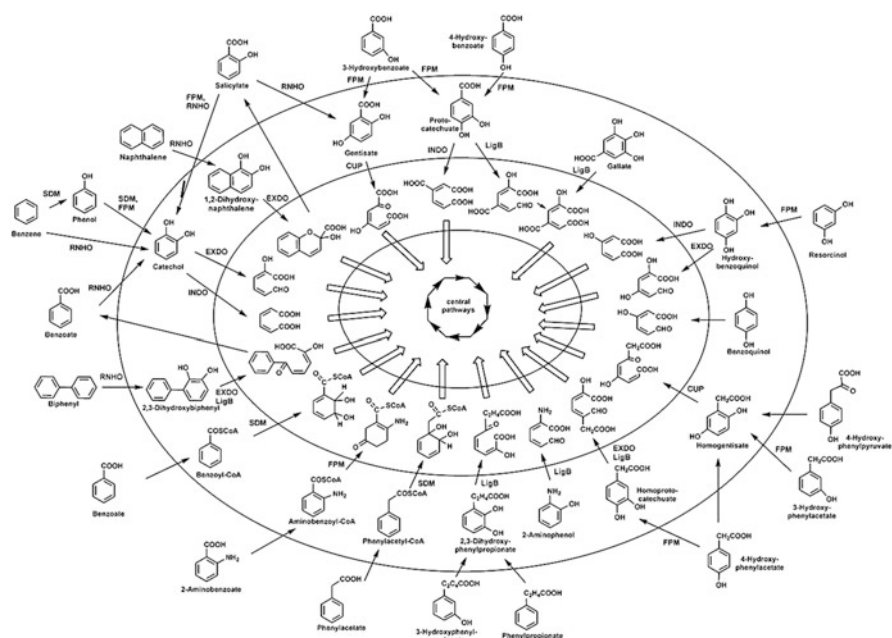


Fig. 9.2 Convergence of catabolic pathways of aromatic compounds at catechol, proto-catechuate, gentisate, hydroxybenzoquinol and funneling into central metabolic pathways; FPM, flavoprotein monooxygenases; RNHO, Rieske non-heme iron oxygenases (specific dihydrodiol dehydrogenases are not indicated); and SDM, soluble diiron monooxygenases. Alternatively, aromatics can be activated by CoA ligases and dearomatization catalyzed by FPM or SDM. Central di- or trihydroxylated intermediates undergo ring cleavage by intradiol (INDO) or extradiol (EXDO) dioxygenases, the LigB superfamily (LigB), or the cupin superfamily (CUP). Products of ring cleavage are channeled to central catabolic reactions (*hollow arrows*) Vilchez-Vargas et al. (2010)

they disrupt cell membrane. *Pseudomonas putida* is known to develop tolerance to solvents by undergoing modifications in the cell membranes. Even catechol and its derivatives have been found to be toxic to other bacteria in the niche affecting microbial community (Schweigert et al. 2001; Guerin 2008).

Degradation of pollutants involves oxygenation of the substrate by oxygenases, which incorporate one or two atoms of oxygen into the organic molecule. Alkanes are oxidized to carboxylic acid and further metabolism occurs via β -oxidation. Aromatic ring structured molecules are oxygenated to form diols and then cleavage of ring results in formation of catechol which is further metabolized to products that enter TCA cycle (Fig. 9.2) Vilchez-Vargas et al. 2010.

A brief discussion on degradation of the important pollutants by *Pseudomonas* follows:

9.4.1 Chlorinated Aliphatics

Trichloroethylene (TLE) is extensively used as a solvent and degreasing agent. It has been encountered in underground water and has been recognized as a priority pollutant by EPA, USA. *Pseudomonas cepacia* and *P. putida* have been characterized for metabolism of TCE, which undergoes cometabolism when toluene, phenol, and nonaromatic compounds are being metabolized as primary substrates. On the contrary reductive dechlorination operating under anaerobic conditions is more efficient (Pant and Pant 2010). But both require the presence of a co-substrate as a carbon and energy source. Chlorinated aliphatic hydrocarbons (CAHs), viz., carbon tetrachloride (CT), tetrachloroethylene (PCE), and trichloroethylene (TCE), contaminating groundwater undergo abiotic and biotic transformation. The products formed are chloroform (CF), methylene chloride (MC), *cis*-1,2-dichloroethylene (c-DCE), *trans*-1,2-dichloroethylene (t-DCE), vinyl chloride (VC), 1,1-dichloroethane, and chloroethene. Addition of phenol at 500 µg/l as a primary substrate resulted in 87 % removal of TCE, while a concentration of 1000 µg/l showed inhibitory effect as TCE removal was reduced to 75 %.

A revertant strain G4-5223-PR1 of Tn5 insertion mutant of *P. cepacia* G4 has been reported to degrade TCE constitutively without aromatic induction (Shields and Reagin 1992). Parent strain of *P. cepacia* G4 is known to grow on phenol and produce catabolic enzymes required for both phenol and TCE degradation (Folsom et al. 1990).

In the case of *Pseudomonas putida* F1, it has been shown that aromatic compounds do not only induce TCE degradation, but toluene dioxygenase is involved in degradation of TCE by *P. putida* F1. All the TCE-negative strains were defective in *todC* which encodes the oxygenase component of toluene dioxygenase required for TCE metabolism by *P. putida* F1. *Pseudomonas putida* F1 also oxidize TCEs and dichloroethylenes and substituted benzenes and 3-methyl-cyclohexene as well as fused and heterocyclic ring systems.

Toluene-oxidizing *P. cepacia* G4 (*Burkholderia cepacia* G4) which expresses toluene 2-monooxygenase showed higher rates of TCE degradation when glucose, acetate, or ethanol was supplied as additional growth substrates. This was attributed to additional supply of NADH that is consumed in a cometabolic degradation of TCE (Kim et al. 2014). Poplar leaf homogenate which contains natural phenolic compounds also induced toluene ortho-mono oxygenase in *B. cepacia* G4 enabling it to grow and degrade TCE (Kang and Doty 2014).

9.4.2 Aromatic Compounds: BTEX

Aromatic compounds are major environmental pollutants which are released in abundance and exhibit properties of being carcinogenic, mutagenic, and teratogenic in nature; above that, these are not readily degraded and tend to persist for long. Plant biomass comprising of lignin is a major contributor of aromatics, and its degradation and recycling is vital in the carbon cycle. Aromatics exist as single ring

structures or as polycyclic aromatic hydrocarbons and are very stable compounds and relatively resistant to microbial degradation (Harwood and Parales 1996). Benzo[a]pyrene, a potential carcinogen, has been recognized as a priority pollutant (EPA; www.epa.gov). Both aerobic and anaerobic bacterial species with capacity to degrade aromatics have been isolated and characterized (Clarke and Ornston 1975; Cao et al. 2009), *Pseudomonas* and allied species are efficient and predominant as they have evolved the necessary genes and enzymes especially oxygenases for initial attack on these relatively resistant compounds for microbial degradation.

Thus majority of the aromatics are funneled to catechols, protocatechuate, and gentisate, and reactions are generally referred as upper pathways. The cleavage products of these are further degraded by lower pathway to enter the central metabolic pathway (Whitley and Lee 2006).

Degradation of BTEX Pollution by petrochemicals benzene, ethylbenzene, toluene, and xylene, referred to as BTEX, ranks next to trichloroethylene in USA and results from spillage, leaking of pipelines, and underground tanks. Benzo[a]pyrene is recognized as a priority pollutant by EPA. Catechol is the key intermediate for degradation of aromatic compounds by fluorescent pseudomonads (*P. aeruginosa*, *P. putida*, and *P. fluorescens*). The catechol can undergo ortho-cleavage or meta-cleavage involving enzymes catechol 1,2 dioxygenase and 2,3-dioxygenase, respectively. Strains of *P. putida* capable of degrading catechol by meta-cleavage also possess genes for the ortho pathways. The expression of alternate pathways is determined by the nature of the aromatic precursors on which the organism grows (Table 9.3). For example, in naphthalene-grown cells, meta-cleavage pathway is induced by naphthalene or salicylate, but the ortho pathway is induced in benzoate grown cells. *Pseudomonas putida* grown on benzoate induce catechol 1,2-dioxygenase, *P. putida* grown on salicylate and phenol induce catechol 2,3-dioxygenase, while *P. putida* mt-2 carries out meta-cleavage enzyme even when grown in absence of aromatic compound. In *P. putida* and *P. aeruginosa* which metabolize catechol by ortho-cleavage, the enzyme catechol 1,2 dioxygenase is induced by *cis*, *cis*-muconate, the product of catechol metabolism and not by catechol itself. *Cis*-*cis*-muconate is also an inducer of two other enzymes of the catechol branch of α -ketoadipate pathway. The enzymes of the metapathway are induced by primary substrates, e.g., salicylate, phenol, *o*-, *m*-, or *p*-cresol, and derivatives of phenol and benzene sulfonate. Thus the metapathway seems to have much less specific control than the induction of ortho pathway. Ortho-pathway is inducible only by catechol and not by 3-methyl catechol or 4-methyl catechol.

Aerobic degradation of BTEX by *Pseudomonas* proceeds via five different pathways (Fig. 9.3). Initial hydroxylation may occur at ortho (Shields et al. 1989), meta (Khomenkov et al. 2008), or para (Whited and Gibson 1991) positions, or on methyl group (Worsey and Williams 1975) or with dioxygenation at 2,3 positions (Gibson and Parales 2000).

In the first step, hydroxylation of the aromatic ring takes place by toluene dioxygenation (TOD) toluene *o*-monooxygenation (TOM), toluene

Table 9.3 BTEX degrading strains of *Pseudomonas* sp.

S. No.	<i>Pseudomonas</i> strain	Substrate	Comments	References
1	<i>Pseudomonas</i> sp. strain CFS-215	Benzene, toluene and <i>p</i> -xylene	Enhanced degradation of benzene and xylene in the presence of toluene	Alvarez and Vogil (1991)
2	<i>Pseudomonas</i> sp. B ₁	Benzene, toluene and <i>p</i> -xylene	Cometabolism of <i>p</i> -xylene Cometabolism of benzene	Yadav and Reddy (1993)
3	<i>Pseudomonas</i> PP01	Benzene, toluene and <i>p</i> -xylene	Cometabolism of xylene	Oh et al. (1994)
4	<i>Pseudomonas</i> sp. D8	Benzene, toluene and phenols	Degradation of benzene and toluene	Chang et al. (1997)
5	<i>P. putida</i> F1	Benzene, toluene and phenol	Individual substrate degradation	Reardon et al. (2000) Parales et al. (2000)
6	<i>P. putida</i> F1	Benzene and toluene	Effect of oxygen on growth rate	Alagappan and Cowan (2004)
7	<i>P. putida</i> F1, <i>P. putida</i> PaW15 <i>P. mendocina</i> KR1, <i>R. pickettii</i> PK01, <i>B. cepacia</i>	Toluene	Chemotaxis for benzene and toluene	Parales et al. (2000)
8	<i>Thermus aquaticus</i> ATCC 25104 <i>Thermus</i> sp. ATCC27978	Benzene, toluene, ethyl benzene <i>o</i> -, <i>m</i> -, <i>p</i> - xylene	Degradation of BTEX at 60C and 70C	Chen and Taylor (1995)

m-monooxygenation (TBU), and toluene *p*-mono-oxygenation. However, each of the pathways does not metabolize all the BTEX compounds; the dioxygenase has broad specificity but metabolizes xylenes only partially. The three monooxygenases are very specific and have limited activity. Molecular oxygen is required both at the initial oxidation and the ring cleavage step and catechol and protocatechuate are the key intermediates formed by *Pseudomonas* (Cao et al. 2009).

Among BTEX, toluene is most easily degraded followed by *p*-xylene, *m*-xylene, benzene, ethyl benzene, and *o*-xylene. Benzene and phenol are also used as carbon and energy sources for cometabolism of compounds like chlorophenol (Li and Loh 2006). The dioxygenases react with aromatic ring and produce a diol, while the monooxygenases produce arene oxides which are unstable and converted into phenols (Mitchell et al. 2002) which are then converted into dihydroxy compounds. *Pseudomonas stutzeri* OX1 degrades *o*-xylene which is considered most calcitrant and produce 3,4-dimethyl catechol which is cleaved by meta-cleavage pathway. Other aerobic bacteria degrading simple aromatic compounds are *P. putida* F1, *Ralstonia* sp. PHS1, *Rhodococcus* sp. *Pseudoxanthomonas spadix* BD-a59, and *Acinetobacter* sp. B-113.

Bacteria producing dioxygenase to catalyze the first step are fast growing consortia comprising of *P. putida* PPO1 and *P. putida* AT33015 which complement

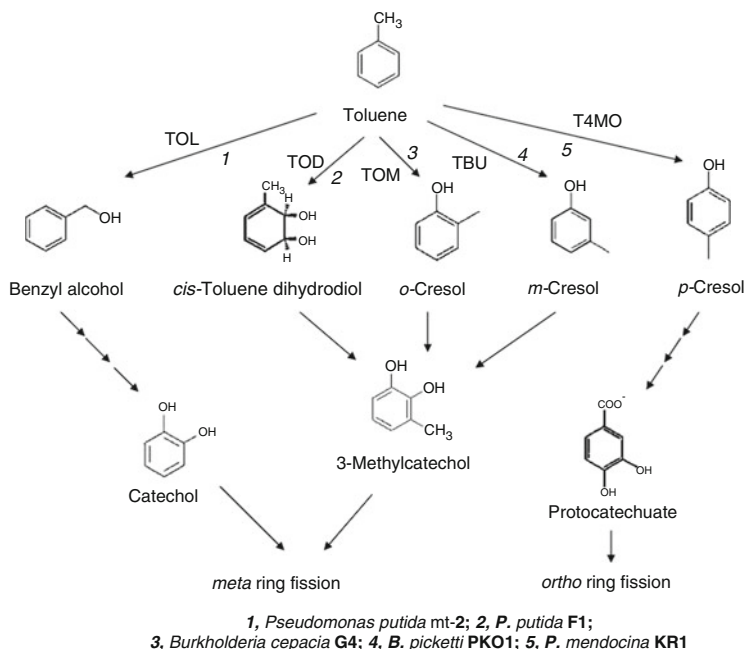


Fig. 9.3 Degradation of toluene by five different pathways carried out by five different strains of *Pseudomonas* and *Burkholderia*. The first step differs in these: TOL involves oxidation of the alkyl substituent resulting in the formation of benzyl alcohol; TOD, involves hydroxylation for ring cleavage by toluene dioxygenase; TOM, involves toluene *o*-monooxygenase; TBU, toluene *m*-monooxygenase; and TMO, toluene *p*-monooxygenase

each other in metabolic activity and can degrade benzene, toluene, and *p*-xylene. The consortium was advantageous in complete degradation of aromatic compounds. A strain of *P. fluorescens* has been isolated which shows nitrate-dependent BTEX metabolism under hypoxic conditions. Such strains will be useful for bioremediation under low oxygen tension such as aquifers (Mikesell et al. 1993).

Other groups of simple aromatic compounds are phenylacetic acid, styrene, phenylethanol, and phenylacetaldehyde, etc. and are assimilated by *P. putida* U, *Flavobacterium* sp., *Escherichia coli*, *Acinetobacter*, etc. These are degraded by formation of acetyl-CoA derivatives and do not involve any oxygenase enzyme. The phenylacetic acid pathway is considered a hybrid of aerobic/anaerobic pathway (Ferrandez et al. 1998). The phenyl-acetyl-CoA has been identified and has various biotechnological applications such as biosynthesis biotransformation and bioremediation (Luengo et al. 2001).

Toluene/benzene is the best-studied pathway in *P. putida* F1 and ten genes, *todC1C2BADEGIH* involved in toluene degradation have been identified. The nucleotide sequence of *tod* genes are similar to corresponding *bph* genes coding

for biphenyl pathway of *P. pseudoalcaligenes* strain KF707. Genomes of BTEX degrading bacteria *P. monteleii* SB3078 and SB3101 have been sequenced (Dueholm et al. 2014).

Bioremediation has been used as an effective technology to reduce the concentration of hydrocarbons in soil and underground water (Dan and Edward 2001). The combination of microorganisms and nutrients were injected into groundwater contaminated with hydrocarbons, and in 5 months 54 % decrease in benzene concentration in groundwater was observed and after 7 months the values were 84 %. Total BTEX reduction of 44 % was observed in 5 months. The organic compounds were degraded by microbial action and the contaminants were converted into less toxic intermediates.

9.4.3 Degradation of Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are the compounds comprising of two or more benzene rings. PAHs are found in the environment after disposal of coal processing wastes, petroleum sludge, asphalt, creosote, and other wood preservative wastes. These have low solubility and persist in the environment for long. Decontamination is necessary because some of these are toxic, mutagenic, and carcinogenic (Patnaik 1992). Sixteen of PAH compounds have been identified as priority pollutants by EPA (Keith and Telliard 1979). These include naphthalene, anthracene, phenanthrene, pyrene, etc. Besides *Pseudomonas* other aerobic bacteria degrade PAHs (Table 9.4). Many bacteria capable of degrading PAHs have been isolated from sites contaminated with coal tar. Jeon et al. (2003) has identified

Table 9.4 Degradation of polycyclic aromatic hydrocarbons (PAH) by *Pseudomonas* and allied aerobic bacteria

PAH	Degrading bacteria
Naphthalene	<i>P. putida</i> , <i>P. fluorescens</i> , <i>Pseudomonas</i> sp., <i>P. aeruginosa</i> , <i>P. paucimobilis</i> , <i>P. vesicularis</i> , <i>P. cepacia</i> (<i>Burkholderia cepacia</i>), <i>P. testosteroni</i> H (<i>Comamonas testosteroni</i> H), <i>Acinetobacter calcoaceticus</i> , <i>Alcaligenes denitrificans</i>
Acenaphthylene	<i>P. putida</i> , <i>P. fluorescens</i> , <i>B. cepacia</i> , <i>Pseudomonas</i> sp.
Anthracene	<i>P. putida</i> , <i>P. paucimobilis</i> , <i>B. cepacia</i> , <i>Flavobacterium</i> sp. <i>Arthrobacter</i> sp.
Phenanthrene	<i>P. putida</i> , <i>C. testosteroni</i> strains H, GZ38A, GZ39, GZ42, <i>P. paucimobilis</i> , <i>Alcaligenes faecalis</i> , <i>Alcaligenes denitrificans</i> , <i>Acinetobacter</i> sp.
Fluoranthene	<i>P. putida</i> , <i>P. paucimobilis</i> , <i>P. cepacia</i> , <i>Pseudomonas</i> sp., <i>Alcaligenes denitrificans</i>
Pyrene	<i>Alcaligenes denitrificans</i> , <i>Rhodococcus</i> , <i>Mycobacterium</i>
Chrysene	<i>Rhodococcus</i> sp.
Benz[a]anthracene	<i>P. putida</i> , <i>Alcaligenes denitrificans</i>
Benzo[a]pyrene	<i>Beijerinckia</i> sp., <i>Mycobacterium</i> sp.

active population in PAH degradation using C^{13} label technique. Polycyclic aromatic compounds having 2–4 rings are used as growth substrates by bacteria and fungi and three main pathways have been characterized (Cerniglia 1992). The pathway involving mono- or dioxygenases are of particular interest and a number of strains of *Pseudomonas* carry out complete degradation of naphthalene and other PAHs (Yang et al. 1994; Ferrero et al. 2002).

Metabolic pathway of naphthalene degradation by *Pseudomonas putida* has been extensively studied, and plasmid-carrying genes for naphthalene and salicylate degradation have been characterized (Dunn and Gunsalus 1973; Yen and Gunsalus 1982, 1985, Peng et al. 2008). While *Pseudomonas fluorescens* and *P. alcaligenes* are known to degrade naphthalene with the formation of gentisate instead of salicylate.

PAHs are hydrophobic and have low bioavailability. Their bioavailability increases with rise in temperature due to higher solubility, diffusion, and reaction rates (Tabak et al. 2003). Naphthalene metabolism is the model sequence of PAH degradation by *P. putida* (Davies and Evans 1964). The metabolic pathway of naphthalene is split into upper pathway, i.e., naphthalene to salicylate, and the lower pathway, i.e., salicylate to TCA cycle intermediates. The first reaction was catalyzed by naphthalene dioxygenase (NahA) to form *cis*-1,2-dihydroxy-1,2-dihydronaphthalene or *cis*-naphthalene dihydrodiol. Naphthalene 1,2-dioxygenase comprises of ferredoxin reductase, ferredoxin, and an iron sulfur protein. Then *cis*-naphthalene dihydrodiol is converted into 1,2-dihydroxynaphthalene by enzyme *cis*-naphthalene dihydrodiol dehydrogenase (NahB). The enzyme 1,2-dihydroxynaphthalene dioxygenase (NahC) catalyzes meta-cleavage of 1,2-dihydroxynaphthalene and the ring cleavage product recycles spontaneously to form 2-hydroxy-2H-chromene-2-carboxylic acid. 2-hydroxy-2H-chromene-2-carboxylic acid under the action of an isomerase and a hydratase-aldolase is converted into semialdehyde through the mediation of *trans*-*o*-hydroxybenzylidene pyruvic acid (Fig. 9.4). The isomerase is 2-hydroxy-2-H-chromene-2-carboxylate isomerase (NahD) and aldolase is *trans*-*o*-hydroxybenzylidenepyruvate hydratase-aldolase (NahE). Salicylaldehyde dehydrogenase (NahF) converts salicylaldehyde into salicylic acid.

The salicylate is further metabolized via catechol (Cerniglia 1992). Genes and enzymes of naphthalene are listed in Table 9.5.

Degradation of phenanthrene and anthracene proceeds via analogous enzymatic reactions, and end products of the upper pathways are 1-hydroxy-2-naphthoic acid and 2-hydroxy-2-naphthoic acid, respectively. 1-hydroxy-2-naphthoic formed from phenanthrene is further metabolized by two pathways yielding salicylic acid and protocatechuate, which are further metabolized yielding intermediates entering TCA cycle. Anthracene which yields 2-hydroxy-3-naphthoic acid as an intermediary product is further metabolized to yield salicylate and catechol (Fig. 9.5).

The catabolic genes for naphthalene degradation in *P. putida* G7 are organized in three operons on 83 kb NAH7 plasmid. One encodes for enzymes of upper

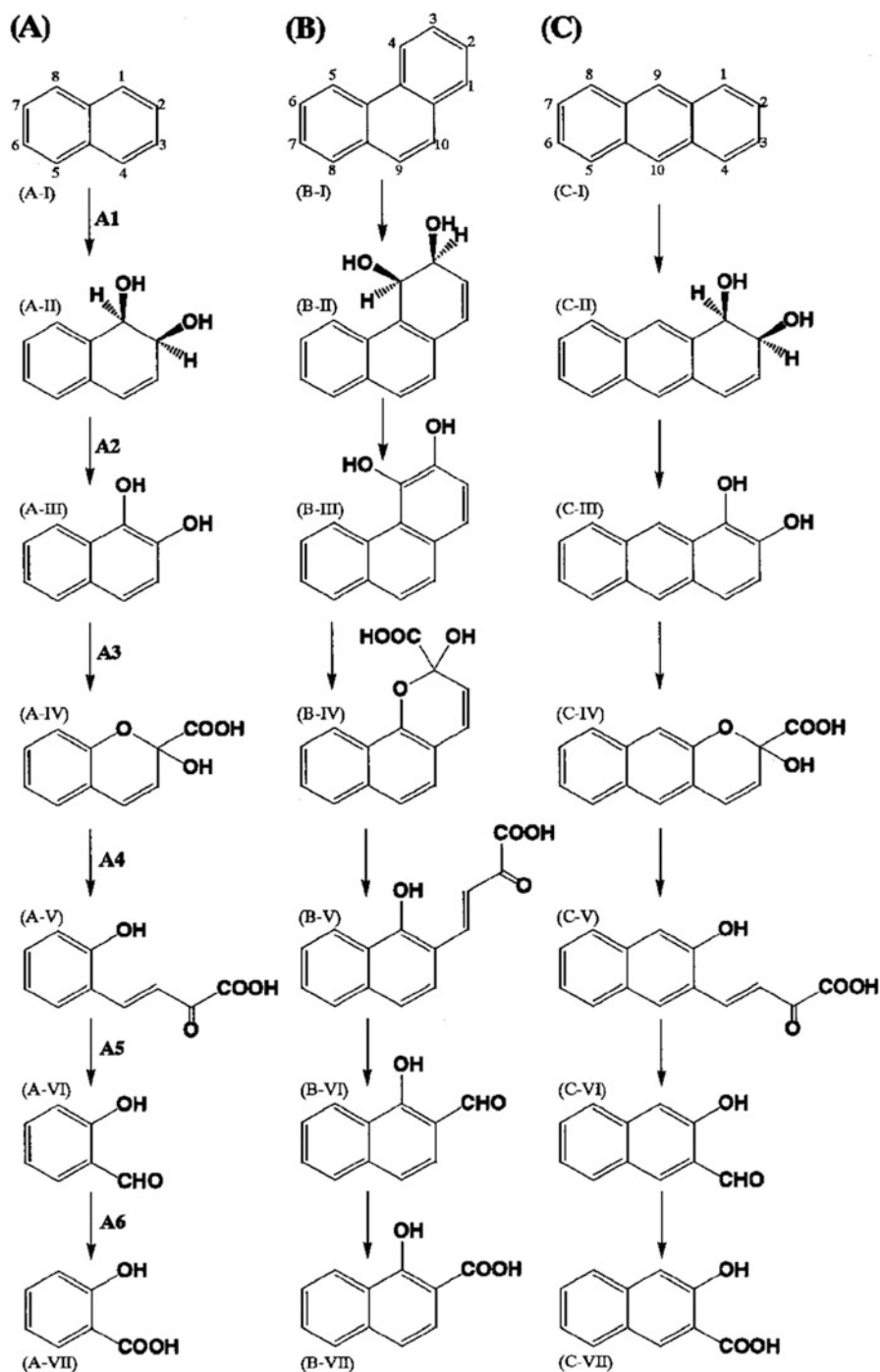


Fig. 9.4 Degradative pathways of naphthalene (a), phenanthrene (b), and anthracene (c) by aerobic bacteria. Enzymes for naphthalene degradation are A1, naphthalene dioxygenase (Nah

Table 9.5 Genes and enzymes of naphthalene degradation encoded on the NAH7 plasmid of *Pseudomonas putida* G1 (Obeyori and Salam 2010)

Substrate	Gene	Encoded protein or function
Naphthalene (upper pathway)	<i>nahAa</i>	Reductase
	<i>nahAb</i>	Ferredoxin
	<i>nahAc</i>	Iron sulfur protein large subunit
	<i>nahAd</i>	Iron sulfur protein small submit
	<i>nahB</i>	<i>cis</i> -naphthalene dihydrodiol dehydrogenase
	<i>nahF</i>	Salicylaldehyde dehydrogenase
	<i>nahC</i>	1,2-dihydroxynaphthalene oxygenase
	<i>nahE</i>	2-Hydroxybenzalpyruvate aldolase
	<i>nahD</i>	2-Hydroxychromene-2-carboxylate isomerase
Salicylate (lower pathway)	<i>nahG</i>	Salicylate hydroxylase
	<i>nahT</i>	Chloroplast-type ferredoxin
	<i>nahH</i>	Catechol oxygenase
	<i>nahI</i>	2-Hydroxymuconic semialdehyde dehydrogenase
	<i>nahN</i>	2-Hydroxymuconic semialdehyde dehydrogenase
	<i>nahL</i>	2-Oxo-4-pentenoate hydratase
	<i>nahO</i>	4-Hydroxy-2-oxovalerate aldolase
	<i>nahM</i>	Acetaldehyde dehydrogenase
	<i>nahK</i>	4-Oxalocrotonate decarboxylase
Regulator for both operons	<i>nahJ</i>	2-Hydroxymuconate tautomerase
	<i>nahR</i>	Induced by salicylate

metabolic pathway, i.e., naphthalene to salicylate; second encodes the lower pathway, i.e., the enzymes involved in conversion of salicylate to TCA cycle intermediates via meta-cleavage pathway. The third operon encodes a regulatory protein, NahR (Yen and Gunsalus 1985; Habe and Omori 2003). NahR protein regulates both upper and lower pathways as a positive control regulator. The *nah* genes are induced by salicylate and NahR is required for their high-level expression (Schell 1986; Fuenmayor et al. 1998).

NAH plasmids pWW60, pDTG1, and pKA1 from *P. putida* NC1B9816, *P. putida* NC1B9816-4, and *P. fluorescens* 5R, respectively, have been found to be similar to NAH7. Gene organization and sequence similarity (about 90 %) was found to be similar in *P. putida* NC1B9816-4, *Pseudomonas* sp. C18, *P. putida* OU582, *P. aeruginosa* PaK1, *P. putida* BS202, and *P. stutzeri* AN10 (Bosch

Fig. 9.4 (continued) AaAbAcAd); A2, *cis*-dihydroxynaphthalene dihydrodiol dehydrogenase (Nah B); A3, 1,2-dihydroxynaphthalene dioxygenase (NahC); A4, 2-hydroxy-2H-chromene-2-carboxylate isomerase (NahD); A5, *trans*-o-hydroxybenzylidenepyruvate hydratase-aldolase (NahE); and A6, salicylaldehyde dehydrogenase (NahF) resulting in the formation of salicylic acid. Phenanthrene and anthracene by parallel series of enzymes are broken down to 1-hydroxy-2-naphthoic acid, which is further metabolized to salicylic acid or protocatechuic acid

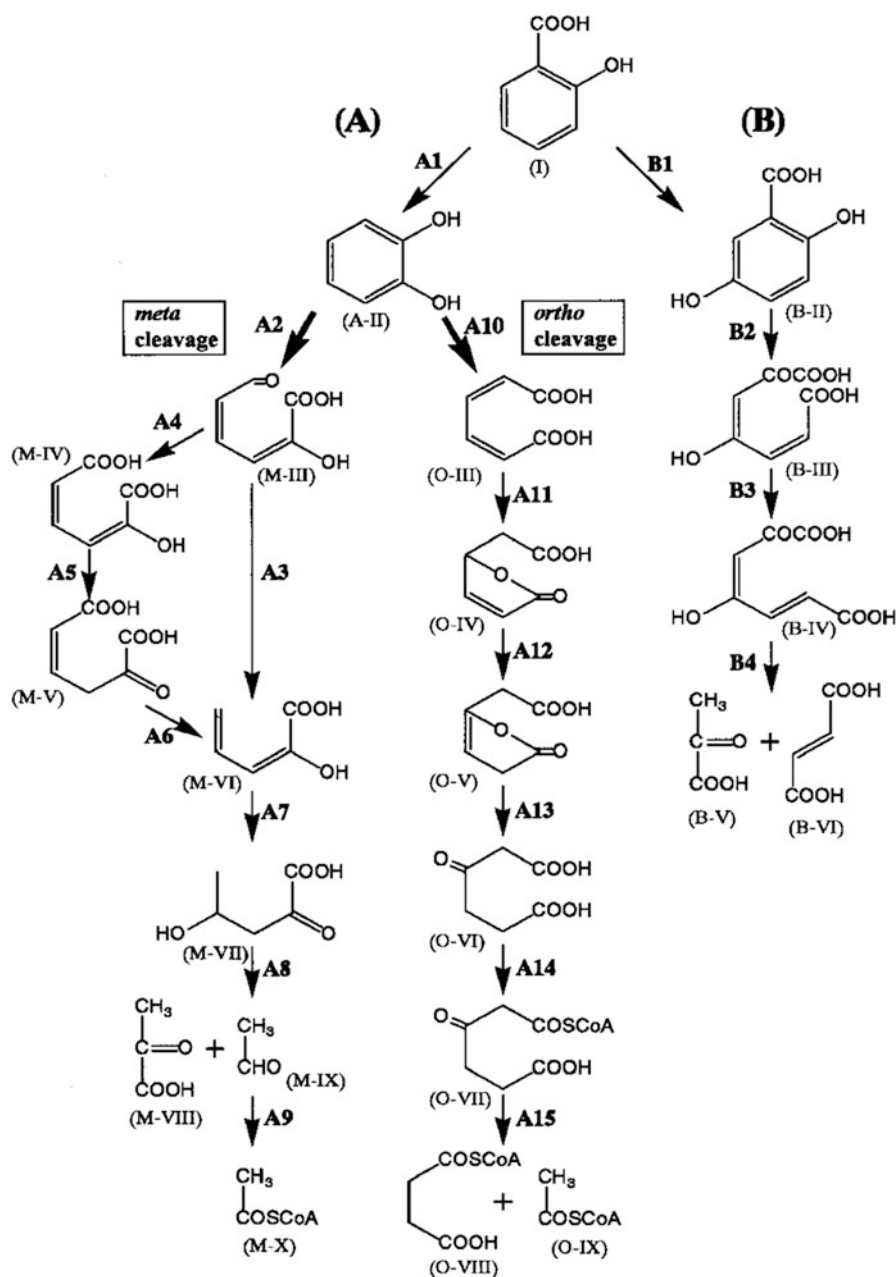


Fig. 9.5 Catabolic pathway of salicylic acid degradation via catechol and gentisic acid. This is also referred as lower catabolic pathway of naphthalene degradation. The sequence of reactions via catechol is under the Nah operon and the ring cleavage of catechol may be in the ortho- or meta-position. The gentisic acid branch is catalyzed by Nag operon. Enzymes of the meta-cleavage are A1, salicylate hydroxylase (NahG); A2, catechol 2,3 dioxxygenase (NahH); A3, hydroxymuconic semialdehyde hydrolase (NahN); A4, hydroxymuconic semialdehyde dehydrogenase (NahI); A5,

et al. 2000). The genes in upper pathway operon and lower pathway operon are organized in sequence, *nahAa*, *Ab*, *Ac*, *Ad*, *BFCQED*, and *nah GTHIN2OMKJ*, respectively.

9.4.4 Degradation of Chlorinated Aromatic Compounds

Chlorinated organic compounds in the natural environment are relatively persistent and non-biodegradable. The carbon–chloride bond is very stable and thus renders the polychlorinated compounds difficult to degrade (Häggbloom and Bossert 2003; Lee et al. 1998). The turnover of chlorinated aromatic pollutants depends upon their susceptibility to microbial transformation (Bhatt et al. 2007). *Pseudomonas* and some other aerobic bacteria have been identified to metabolize highly chlorinated compounds such as chloroalkanes (Hardman 1991), chlorobenzenes (Guerin 2008), chlorobenzoates (Olaniran and Igbinsosa 2011), chlorophenols (Field and Sierra-Alvarez 2008), and polychlorinated biphenyls. Halogen moiety is removed either by the specific dehalogenase or as part of the oxidation of chlorocatechol.

Chlorinated aromatic compounds including chlorobenzene, chlorobenzoates, chlorophenols, chloroanilines, hexachloro-benzene, polychlorinated biphenyl, chloronitrophenol, etc. are major pollutants of the environment and are used in manufacture of dyes, drugs, pesticides, and other industrial products. These have been released into the environment in large quantities in the form of herbicides, pesticides, etc. The stability of these compounds depends upon the carbon–chlorine bond; it is therefore obvious that for their degradation the C–Cl bonds be cleaved. This can happen in two ways (1) spontaneous dechlorination of an unstable intermediate of unrelated enzymatic reactions and (2) by an enzymatic dechlorination where the C–Cl cleavage is catalyzed by a dechlorinase (Fetzner and Lingens 1994; Kao et al. 2005).

Enzymatic dehalogenation, i.e., removal of chlorine atom takes place either by hydrolytic, reductive, or oxygen-dependent dehalogenation reaction. Hydrolytic removal of chloride ion involves the replacement of chlorine by hydroxyl group in the aromatic ring. Reductive process of dehalogenation involves replacement of chlorine by hydrogen atom (Arora and Bae 2014). In oxygenolytic dehalogenation, chlorine and this atom are replaced by a hydroxyl group containing an oxygen atom

Fig. 9.5 (continued) 4-oxalocrotonate isomerase (NahJ); A6, 4-oxalocrotonate decarboxylase (NahK); A7, 2-oxopent-4-enoate hydratase (NahL); A8, 2-oxo-4-hydroxypentanoate aldolase (NahM); A9, acetaldehyde dehydrogenase (NahO); Enzymes of ortho cleavage are A10, catechol 1,2-dioxygenase; A11, *cis*, *cis*-muconatelactonizing enzyme; A12, muconolactone isomerase; A13, β -ketoadipate-enol-lactone hydrolase; A14, β -ketoadipate:succinyl-CoA transferase; and A15, β -ketoadipylCoA thiolase. The end products of meta-cleavage are pyruvic acid (M-VIII) and acetyl-CoA (M-X) and that of ortho-cleavage are succinyl-CoA (O-VIII) and acetyl-CoA (O-IX). For gentisic acid pathway the enzymes are B1, salicylate 5-hydroxylase (NagGHAaBb); B2, gentisate 1,2-dioxygenase (NagI); B3, maleylpyruvate isomerase (NagL); and B3 fumarylpyruvate hydrolase (NagK). End products formed are pyruvic acid (B-V) and fumaric acid (B-VI)

derived from oxygen. This may involve a monooxygenase or a dioxygenase. 4-chlorobenzoyl-CoA dehalogenase involved in 4-chlorobenzoate degradation is the best-studied dehalogenase which catalyzes conversion of 4-chlorobenzoyl-CoA to 4-hydroxybenzoyl-CoA. Enzymes have been found in many aerobic bacteria including *Pseudomonas* sp. CBS3 (now *Burkholderia* sp. CBS3). A monooxygenase enzyme is involved in oxidative dehalogenation of pentachlorophenol, chlorophenol, and 2,4,6-trichlorophenol. A number of specific dehalogenase have been characterized in *Sphingomonas* sp. UG30, *Pseudomonas cepacia* AC100 (now *Burkholderia cepacia*), *Ralstonia eutropha* (*Cupriavidus necator*), and other aerobic bacteria such as *Azotobacter* (Table 9.6).

Chlorinated aromatic compounds undergo biodegradation and the compounds containing O₂ as functional group, e.g., chlorophenols and chlorobenzoates, behave differently than the compounds not having the functional oxygen such as chlorobenzene (CB), polychlorinated benzenes (PCB), and chlorinated dioxins (CDD).

9.4.4.1 Chlorobenzenes

Chlorobenzenes include chlorobenzenes (CB), dichlorobenzenes (DCB), trichlorobenzene (TCB), tetrachlorobenzene (TeCB), pentachlorobenzene, and hexachlorobenzenes. Chlorobenzenes are used as industrial intermediates and solvents. A number of strains of *Pseudomonas* and allied aerobic bacteria *Burkholderia*, *Sphingomonas*, etc. have been identified for degradation and utilizing benzene as sources of carbon and energy (Table 9.7).

Aerobic degradation of chlorobenzene proceeds by initial cleavage by a dioxygenase to chlorocatechol through chloro-*cis*-dihydrodiol. Chlorocatechol is further metabolized either by ortho-cleavage or meta-cleavage. In both cases the chloride ion is removed in the first step by a dehalogenase (Reineke and Knackmuss 1984; Vogt et al. 2004a, b) or is not removed and end up forming dead-end products which are not further metabolized.

9.4.4.2 Chlorophenols

Chlorophenols are comprised of isomers, chlorophenol (CP) dichlorophenol (DCP), trichlorophenol (TCP), tetrachlorophenol (TeCP), and pentachlorophenol (PCP). The technical grade PCP is 87–89 % PCP, 6 % other chlorophenols and 3 % phenoxyphenols and traces of other chlorinated aromatics. They have been extensively used as biocides. 4-CP is used as an antiseptic for home, hospital, and on farms. 2,4-DCP and 2,4,5-TCP were intermediates of 2,4-D and 2,4,5-T synthesis. PCP, TCP, and TeCP are extensively used as wood preservatives against fungi. In the natural environment, the soil microorganisms have been reported to be quite active to degrade various isomers of chlorophenols in soil (Sanchez et al. 2004; Mahmood et al. 2005). Aquifer sediments from PCP-contaminated sites were shown to readily degrade PCP (Kao et al. 2004). Aerobic degradation of chlorophenols has also been observed in surface water. Extensive studies are also available on anaerobic degradation of chlorophenols but will not be discussed here.

Table 9.6 List of different types of dehalogenases involved in the aerobic degradation of chlorinated aromatic compounds (Arora and Bae 2014)

Dehalogenase	Reaction catalyzed
4-chlorobenzoyl-CoA dehalogenase	Converts 4-chlorobenzoyl-CoA to 4-hydroxybenzoyl CoA
4-chlorobenzoate dehalogenase	Converts 4-chlorobenzoate to hydroxybenzoate
Chlorothalonil dehalogenase	Converts 2,4,5,6-tetrachloro- isophthalonitrile (chlorothalonil) to 4-hydroxy-trichloroisophthalonitrile
Atrazine chlorohydrolase	Converts atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine) to 2-hydroxy-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine
Tetrachlorohydroquinone dehalogenase	Converts tetrachlorohydroquinone to 2,6-dichlorohydroquinone via trichlorohydroquinone
2,5-dichlorohydroquinone reductive dehalogenase	Converts 2,5-dichlorohydroquinone to chlorohydroquinone (CHQ)
Chlorohydroquinone dehalogenase	Dehalogenates 2-chlorohydroquinone to hydroquinone, involved in the degradation pathway of 2-chloro-4-nitrophenol and 4-amino-2-chlorophenol
4-chloro-2-aminophenol dehalogenase	Dehalogenates 4-chloro-2-aminophenol to aminophenol
Pentachlorophenol 4-monooxygenase	Converts pentachlorophenol to tetrachlorohydroquinone
Chlorophenol 4-monooxygenase	Converts 2,4,5-trichlorophenol to 2,5-dichloro- <i>p</i> -hydroquinone
2,4,6-trichlorophenol (TCP) monooxygenase	Converts 2,4,6-TCP to 2-chloro- hydroxyquinone via 2,6-dichloro- quinone
4-chlorophenylacetate dioxygenase	Converts 4-chlorophenylacetate to 3,4-dihydroxyphenylacetate
2-halobenzoate dioxygenase	Converts 2-halobenzoate to catechol
Chlorobenzene dioxygenase (Tec A)	Converts 1,2,4,5-tetrachlorobenzene to a 3,4,6-trichlorocatechol

Table 9.7 Important strains of *Pseudomonas* and related bacteria degrading chlorobenzenes

1.	Chlorobenzene (CB)	<i>Pseudomonas aeruginosa</i> RH01, <i>P. putida</i> GJ31 <i>Pseudomonas</i> sp. strain JS100, JS150, JS6 <i>Pseudomonas</i> sp. <i>Pseudomonas veronii</i> B549, 1347 <i>Ralstonia</i> sp. JS 705, <i>Burkholderia</i> sp. strain PS12, PS14, <i>Stenotrophomonas</i> sp.
2.	1,2-DCB	<i>Pseudomonas</i> sp. strain GJ60; JS100; P5 <i>Burkholderia</i> PS12, PS14, <i>Acidovorax avenae</i>
3.	1,3-DCB	<i>Pseudomonas</i> sp. strain P51, <i>Burkholderia</i> PS12, PS14
4.	1,4-DCB	<i>Pseudomonas</i> sp. B1 <i>Pseudomonas</i> sp. strain JS150, JS6, PS51
5.	1,2,4-TCB	<i>Pseudomonas</i> sp. strain P51 <i>Burkholderia</i> sp. PS12, PS14
6.	1,2,3,4-TeCB	<i>Pseudomonas chlororaphis</i> RW71
7.	1,2,4,5-TeCB	<i>Pseudomonas</i> PS12, <i>Burkholderia</i> PS14

Table 9.8 *Pseudomonas* and related bacteria capable of degrading chlorophenols [Modified from Field (2007)]

Isomer	Bacteria
2 CP	<i>Pseudomonas pickettii</i> LD1 (<i>Ralstonia pickettii</i>), <i>Alcaligenes</i> sp. A7-2
3 CP	<i>R. pickettii</i> LD1, <i>Alcaligenes xylosoxidans</i> JH1
4 CP	<i>R. pickettii</i> LD1, <i>Pseudomonas</i> sp. B13, <i>Alcaligenes xylosoxidans</i> JH1, <i>Alcaligenes</i> sp. A7-2, <i>Comamonas testosteroni</i> CPW301
2,4 CP	<i>Pseudomonas</i> sp. DP-4, <i>Pseudomonas</i> sp. strain NC1B9340, <i>Sphingomonas</i> sp. P5
2,4,6 TCP	<i>P. saccharophila</i> ; <i>R. pickettii</i> ; <i>Sphingomonas</i> sp. K74, MT1; <i>R. eutropha</i> JMPB4; <i>Novosphingobium lentum</i> MT-1
2,3,5 TCP	<i>Sphingomonas</i> sp. P5
2,3,4,6 TeCP	<i>P. saccharophila</i> , <i>Sphingomonas</i> sp. P5, <i>Sphingomonas</i> k74 and MT1, <i>Novosphingobium lentum</i> MT1
PCP	<i>Pseudomonas</i> sp. 4G25 and 4G30, <i>Pseudomonas</i> sp. AR2, <i>Pseudomonas</i> sp. strain SR 3, <i>Pseudomonas</i> sp. IST103, <i>P. mendocina</i> NSYSU, <i>Sphingomonas</i> sp. P5, <i>S. chlorophenolica</i> RA2, <i>Novosphingobium lentum</i> MT1

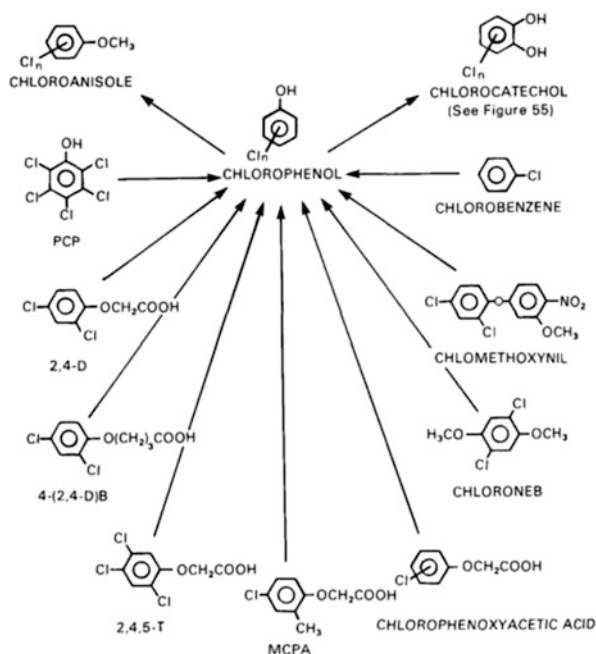
A number of aerobic bacteria with capacity to degrade different isomers of chlorophenols and utilized as sources of carbon and energy have been identified (Field and Sierra-Alvarez 2007). These include strains of genus *Pseudomonas* and allied bacteria (Table 9.8) earlier classified as *Pseudomonas* (*Sphingomonas*, *Ralstonia*, *Sphingobium*, etc.) and other genera such as *Rhodococcus*, *Arthrobacter*, *Alcaligenes*, *Flavobacterium*, *Streptomyces*, *Nocardioide*s sp., etc. (Im et al. 2004). They all mineralized chlorophenol and utilized those as sources of carbon and energy with concomitant production of biomass (Yang et al. 2005).

Lower chlorinated phenols (1–2 chlorine substituents) are initially attacked by monooxygenases yielding chlorocatechols as the first intermediates of chlorocatechol pathway. Chlorocatechols undergo ring cleavage prior to release of chloride ion. The polychlorinated phenols (3–5 chlorine atoms) are converted to chlorohydroquinones as the initial intermediates of hydroquinone pathway. The chlorine is removed in the subsequent steps prior to the ring cleavage (Fig. 9.6).

The chlorocatechol pathway for degradation of 2,4 DCP is initiated by a monooxygenase forming 3,5-dichlorocatechol. Chlorocatechol undergoes ortho-cleavage yielding 2,4-dichloromuconic acid which is converted into 2-chloro-4-carboxymethylene but-2-enolide by the lactonizing enzyme, this is the stage when the first dechlorination takes place. The butenolide is converted to 2-chloromaleylacetic acid which is further metabolized to give succinic acid (Fig. 9.7).

The conversion of chlorocatechols proceeds via ortho-cleavage, while the meta-cleavage alternate pathway is less common because 2,3-dioxygenase is inactivated by 3-chlorocatechols (3CC) and 5 chloro-2-oxymuconic semialdehyde formed by cleavage of 4-chlorocatechol is toxic to bacteria. However, some bacteria are able to use meta-cleavage pathway. A 2,3-dioxygenase has been isolated from *P. putida* GJ31 which can catalyze meta-cleavage of 3CC without the enzyme being inactivated.

Fig. 9.6 Convergence of metabolism of chlorinated aromatic compounds at chlorophenol and further breakdown via chlorocatechol (EPA 1986)



The chlorohydroquinone pathway of pentachlorophenols (PCP) degradation is initiated by hydroxylation in para position resulting in the formation of *p*-tetrachloro-hydroquinone (TeCHQ) (Fetzner 1998). In *Sphingomonas chlorophenolica* ATCC39723, the hydroxylation of PCP takes place by PCP-4-monooxygenase (Pcp B). Similar monooxygenases have also been reported in other aerobic bacteria (Thakur et al. 2002). TeCHQ is sequentially dechlorinated in two steps to 2,6-dichloro-1,4-hydroquinone (2,6-DCHQ) by a reductive dehalogenase (PcpC). 2,6 DCHQ is oxidized to form 2-chloromaleylacetate accompanied with the release of one chloride by the action of 2,6-DCHQ 1,2-dioxygenase (PcpA) (Xun et al. 1999; Xu et al. 1999). Recently, a hydroxyquinone hydratase has been identified in *S. chlorophenolicum* which converts hydroxyl-1,4-quinone to 1,2,4,5-tetrahydroxybenzene which undergoes autooxidation to 2,5-dihydroquinone. Thus hydro-lase may provide an alternate pathway for metabolism of aromatic range.

Cometabolism of chlorophenols is an important aspect of chlorophenol degradation. A strain of *Pseudomonas* isolated from soil with ability to degrade benzene cometabolizes 2-CP and 4-CP to CO₂. Toluene-grown cells of *Pseudomonas putida* showed degradation of 2 CP, 3 CP, 4 CP, 2,4-DCP, 2,3-DCP, 2,5-DCP, 3,4-DCP, and 2,4,5-TCP forming chlorocatechols as intermediates (Spain and Gibson 1988). Methylstyrene also serves as a primary substrate for 2-CP and 4-CP degradation by *P. putida* (Bestetti et al. 1992). Even chlorophenols serve as primary substrate for degradation of other chlorophenols, e.g., *Pseudomonas* sp. B13 degrades 2-CP and 3-CP when growing on 4-CP. PCP serves as primary substrate for degradation of

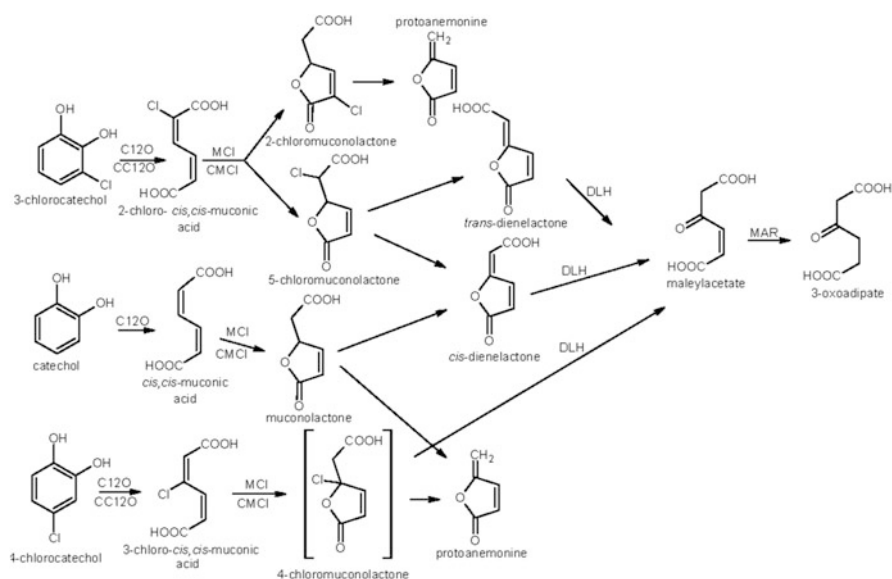


Fig. 9.7 Degradation of catechol and chlorocatechol to 3-oxoadipic acid by ortho-cleavage; C12O, catechol 1,2 dioxygenase; CC12O, chlorocatechol 1,2 dioxygenase; MCI, muconatecyclisomerase; CMCI, chloromuconatecyclisomerase; DLH, dienelactone hydrolase; and MAR, maleylacetate reductase

3,4,5 TCP and 2,3,5,6-TeCP (Liu et al. 1991a, b). PCP degrading *S. chlorophenolica* are able to mineralize 2,3,6-TCP, 2,4,6 TCP, and 2,3,4,6-TeCP (Yang et al. 2005). The primary substrate induces synthesis of dioxygenases or monooxygenases thus facilitating the cooxidation of chlorophenols. Substrates such as sugars can also induce cometabolism though they do not induce dioxygenases or monooxygenases. Wang and Loh (2000) proposed that they may support cometabolism by generating NADH required for dioxygenases.

9.4.4.3 Chlorinated Benzoates

Isomers of the chlorinated benzoic acids are chlorobenzoic acid (CBA), dichlorobenzoic acid (DCBA), and trichlorobenzoic acid (TCBA). Their sources in the environment are their use as herbicides [e.g., 2,3,6-TCBA and dicamba (2-methoxy-3,6-dichlorobenzoate)] or as metabolites of other halogenated compounds such as pentachlorobenzyl alcohol (fungicide) and aerobic degradation of polychlorinated biphenyls or anaerobic degradation of chlorophenols and DDT (Chaudhary and Chaplamadhugu 1991).

Enough evidence is available about the degradation of 2CBA, 3CBA, and 4CBA, and dicamba in soil and natural environment (Kocher and Kahlon 1999; Baggi and Zangrossi 2001; Ramirez-Saad et al. 2000; Wang et al. 2004). Aerobic degradation of chlorobenzoates also occurs in lake water and sewage. A number of strains of *Pseudomonas* sp. have been identified to utilize chlorinated benzoates as

Table 9.9 *Pseudomonas* and allied bacteria growing on chlorobenzoic acids as sources of carbon and energy

S. No.	Compound	Bacteria
1	2CBA	<i>Pseudomonas aeruginosa</i> JB2; <i>P. aeruginosa</i> 142; <i>P. cepacia</i> (<i>Burkholderia cepacia</i>) strain KZ2, 2CBS; <i>B. cepacia</i> , <i>Pseudomonas</i> sp. CPEZ; <i>Pseudomonas</i> sp. P111A; <i>Pseudomonas</i> sp. 13302; <i>P. pickettii</i>
2	3CBA	<i>P. aeruginosa</i> , <i>P. putida</i> DP4, <i>P. aeruginosa</i> JB2; <i>Pseudomonas</i> sp., <i>P. knackmussi</i> B13; <i>Pseudomonas</i> WR912, <i>Pseudomonas</i> sp. P111; <i>Comamonas</i> sp., <i>Ralstonia eutropha</i> JMP134 (<i>Cupriavidus necator</i> JMB134)
3	4CBA	<i>B. cepacia</i> P166; <i>P. paucimobilis</i> BPSI-3; <i>Pseudomonas</i> sp. S-47, DJ-12; <i>Pseudomonas</i> sp. P111; <i>Pseudomonas</i> sp. CBS3
4	2,4-DCBA	<i>P. aeruginosa</i> JB2, <i>Pseudomonas</i> sp. P111A
5	2,4-DCBA	<i>P. aeruginosa</i> sp. 142, <i>R. pickettii</i> , <i>P. fluorescens</i> PNK-3
6	2,5-DCBA	<i>P. aeruginosa</i> JB2, <i>Pseudomonas</i> sp. CPE2, <i>Pseudomonas</i> WR912, <i>Pseudomonas</i> sp. B13
7	2,3,5-TCBA	<i>P. aeruginosa</i> JB2, <i>Pseudomonas</i> sp. P111
8	2,4-D	<i>P. aeruginosa</i> DP5, <i>P. putida</i> DP4

source of carbon and energy. Besides, chlorobenzoates undergo cometabolism by *Pseudomonas* grown on benzoic acid, 4-hydroxy benzoic acid, *o*-anisate, toluate, and benzene.

A number of strains of *Pseudomonas* using chlorobenzoic acids as sources of carbon and energy have been isolated and characterized in our laboratory (Kocher and Kahlon 2003; Saini and Kahlon 1998). Several of the strains degrade chlorobenzoates (Table 9.9). Large diversity was observed for 3-CBA degrading strains as the 150 isolates, which could be distinguished into 48 genotypes on the basis of 16s RNA genes. Several strains also degrade different isomers of dichlorobenzoic acids and trichlorobenzoic acids. Mineralization of chlorinated benzoic acids is evidenced on the basis of stoichiometric release of chloride and growth as the available source of carbon and energy (Kocher and Kahlon 2003; Fulthorpe et al. 1998).

Release of chloride ion has been reported from 2-CBA, 3-CBA, 2,5-DCBA, and 3,5 DCBA (Reineke and Knackmuss 1980; Banta and Kahlon 2007). The biodegradation pathway for 3-CBA is initiated by a dioxygenase-yielding chlorocatechol through the formation of diols. Chlorocatechols are ortho-cleaved to yield chloromuconic acids (Schmidt et al. 1985; Häggblom and Bossert 2003; Muller et al. 1996). The chloride group is released as the chloromuconic acids are lactonized to form either *cis* or *trans* 4-carboxymethylene-but-2-ene-1,4-olides. Meta-cleavage of 3-chlorocatechol is not common as cleavage of 3-chlorocatechol forms 2-hydroxy-6-chlorocarboxy-muconate that inactivates 2,3-chlorocatechol dioxygenase. However, some strains such as *P. putida* GJ-31 have evolved 2,3-dioxygenase that catalyze 3-chlorocatechol degradation without its activity being affected, resulting in release of chloride group (Kaschabek

et al. 1998). The meta-cleavage of 4-chlorocatechol results in the formation of 5-chloro-2-hydroxymuconic semialdehyde, which is oxidized to 5-chloro-2-hydroxymuconic acid and further metabolized to chloroacetate and acetate (Arendorf and Focht 1995). For 3-chlorocatechol degradation, other pathways are available which do not form chlorocatechol as an intermediate.

In one such pathway, chlorine is replaced by hydroxyl group to form 3-hydroxybenzoic acid. Another pathway involves the conversion of 3CBA to protocatechuate (Menn et al. 2010). For 4CBA degradation, most of the soil isolates use hydrolytic pathway where chlorine group is replaced by hydroxyl group to yield 4-hydroxybenzoic acid, whereas only one isolate of the 20 obtained utilized the chlorocatechol pathway (Yi et al. 2000). The hydrolytic pathway uses three enzyme steps to replace chloride group by hydroxyl group. In the first step, the 4CBA is ligated to coenzyme A to form 4-chlorobenzoyl coenzyme A, and in the second step, chloride group is removed by hydrolytic dehalogenase and subsequently thioester is hydrolyzed to liberate coenzyme A (Zhou et al. 2004).

9.4.4.4 Polychlorinated Biphenyls

PCBs have high chemical stability, low water solubility, low toxicity, and low volatility. These are used as insulating liquids for transformers, as grease for vacuum pumps and turbines, as coolants, production of wrapping paper, carbon paper, inks, paints, tires, etc. and their dechlorination is a biological process. PCBs generally occur as mixtures of congeners. The aerobic degradation of PCB has been enhanced by addition of oxygen, cosubstrates, surfactants, and inducers. Presence of biphenyl as a primary substrate enhances cometabolism of PCBs in contaminated soil. Combined treatment of contaminated sediments with biphenyl, oxygen, and nutrients enhanced degradation of Aroclor 1242 in soil. *Arthrobacter* sp. B113 and *Ralstonia eutrophus* H850 induced with carvone and salicylate in presence of surfactant sorbitan trioleate showed 53–59 % removal of PCB in sediments (Singer et al. 2000). Bioreactor packed with beads containing a three-member coculture of *Arthrobacter* sp., *R. eutrophus*, and *Acinetobacter* sp. successfully degraded PCB (Fava and Di Gioia 1998).

Pseudomonas cepacia (now *Burkholderia cepacia*) has been reported to utilize 4CBp, 2CBp, and 3CBp as sole carbon and energy sources. Growth of 2CBp and 3CBp is restricted due to accumulation of toxic intermediates. *B. cepacia* P166 converts 4CBp to 4-chlorobenzoate, which results in formation of 4-chlorocatechol which is further metabolized by a meta-cleavage pathway. Several microorganisms isolated for PCB degradation under aerobic conditions show that less chlorinated PCBs are degraded faster, dioxygenation occurs at the least substituted ring, and PCBs with substituents on both rings are more calcitrant than congeners containing an unsubstituted ring (Leigh et al. 2006; Furukawa and Fujihara 2008). Besides aerobic metabolism, PCBs undergo cometabolism by *Pseudomonas*, *Alcaligenes*, *Achromobacter*, *Burkholderia*, *Comamonas*, *Sphingomonas*, *Ralstonia*, and *Acinetobacter* besides some gram-positive bacteria.

Genes coding for biphenyl metabolic enzymes are clustered as *bph* gene cluster. PCB congeners with double ortho substitutions are poorly degraded probably due to

steric hindrance for their dioxygenase enzyme. *Burkholderia xenovorans* LB400, *B. cepacia* (both formerly *Pseudomonas*), *Pseudomonas* sp. KSS102, and *Ralstonia eutrophus* H850 (*Alcaligenes eutrophus*) display the broadest substrate specificity (Goris et al. 2004).

Anaerobic degradation of PCB takes place by reductive dehalogenation especially in the river bed sediments where the highly chlorinated PCBs are converted into lower chlorinated congeners (Bedard et al. 2006).

9.4.4.5 Chlorinated Dioxins

Dioxins are the pollutants that are formed during incineration of waste, forest fires, and industrial processes such as pulp bleaching and synthesis chemicals including herbicide, 2,4,5-T, hexachlorophene, and pentachlorophenol. There exist 75 polychlorinated congeners of polychlorinated dibenzodioxins (PCDD) and 135 of polychlorinated dibenzofurans (PCDF) which vary in physicochemical properties, bioaccumulation, and toxicity. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is most toxic and is carcinogenic. Human beings absorb foods contaminated with these and these accumulate in fatty tissues and may cause toxic reaction such as pimples, etc.

Aerobic biodegradation of DCDDs is restricted to congeners containing up to four chlorine atoms. *Pseudomonas veronii* PH03 grows on chlorinated dioxin as sources of carbon and energy. *Pseudomonas veronii* PH03 utilizes 1-CDD and 2-CDD by bringing about the ring cleavage of one of the ring and grow on the aliphatic acids generated as a result of ring cleavage and accumulated 3-chlorocatechol (3CC) and 4-chlorocatechol (4CC), respectively, as dead-end products (Hong et al. 2004). Similarly, *Sphingomonas* sp. RW1 grows on 4-CDF and five carbon, 2-hydroxypenta-2,4-dienoate released as a result of ring cleavage is used as a carbon and energy source while 3-chlorosalicylic acid (CSA) accumulates as a dead-end product. Complete mineralization of 2CDF and 3CDF has been achieved by using cocultures of *Sphingomonas* sp. RW16 and *Pseudomonas* RW10. *Pseudomonas* sp. RW10 degrades 5 CSA and 4 CSA which accumulate as a result of degradation of 2 CDF and 3 CDF, respectively (Wittich 1998; Halden et al. 1999). The coculture of *Sphingomonas* sp. RW1 and *Burkholderia* sp. strain JWS completely degrade 4 CDF and the later strain also degrades 3 CSA.

Dioxins also undergo aerobic cometabolism and monochloro- and dichloro-congeners account for 84 % of the reported cases of aerobic bacterial chlorinated dioxin degradation. Trichloro- and tetrachloro- congeners are degraded to a limited extent and molecules with five or more chlorine atoms are not prone to aerobic degradation. For cometabolism of chlorinated dioxins, the nonhalogenated dibenzofuran, dibenzo-*p*-dioxin, biphenyl, *o*-chlorobenzene, and benzoic acid serve as primary substrates (Habe et al. 2001; Hong et al. 2002). The biphenylic dioxin compounds are attacked by angular dioxygenases. The angular dioxygenases catalyze formation of diols which spontaneously form chlorinated 2,2',3-trihydroxydiphenyl ether (THDE) and chlorinated 2,2',3-trihydroxybiphenyl (THB) from PCDD and PCDF, respectively. Subsequent hydrolytic dioxygenases open the ring by meta-cleavage of the dihydroxylated ring structures, chlorinated catechols,

or chlorinated salicylate formed from PCDD or PCDF, respectively. In *Bacillus megaterium* cytochrome P450 plays a role of a monooxygenase for hydroxylation of 2,3-DCDD and 2,3,7-TCDD yielding hydroxylated metabolites of these dioxins (Sulistyaningdyah et al. 2004).

9.4.5 Genetic Basis of Degradative Pathways in *Pseudomonas* sp.

A sudden shift in biology of *Pseudomonas* was observed with the identification of catabolic plasmids and clear taxonomic grouping of *Pseudomonas* by Palleroni based on a comparative study of 16S rRNA sequence (Palleroni et al. 1973). Analysis of the genes coding for specific degradative reaction or sequence proved useful to enhance the metabolic efficiency of microorganisms for specific environmental applications and to engineer cells for desirable catabolic activities using molecular techniques of recombinant DNA technology and mutagenesis (Singh et al. 2008; Wood 2008).

9.4.5.1 Catabolic Plasmids

The plasmids are extrachromosomal genetic elements and possess flexibility of function and mobility among populations as plasmids can be transferred horizontally primarily through the process of conjugation. The genes coding for degradative functions are usually clustered under the control of specific operon whether on the plasmid or on the chromosome. The catabolic gene clusters function as insertion sequences and plasmids as mobile genetic elements. Exposure of a microbial community to hydrocarbons has resulted in gene rearrangement and an increase in the number of plasmids and enhanced metabolic versatility of the host cell. Such genetic potential allows for evolution of integrated and regulated pathways for degradation of pollutants. Catabolic plasmids have been identified to code for a vast array of catabolic functions and play an indispensable role in carbon cycle. They are large measuring between 80 and 200 kb, but some may be mega plasmids with size up to 1000 kb or even more (Top et al. 2002; Schmidt et al. 2011). They attribute the property to degrade and recycle the most complex, most toxic, and most polluting manmade molecules. The plasmid carries one or more clusters of multicistronic units containing up to 10–15 genes. The plasmids are capable of horizontal transfer through conjugation and spread fast among populations. In some cases the gene clusters occur within transposable elements and thus can move between the plasmid and chromosome. Pseudomonads have been able to evolve novel catabolic functions by rearranging existing pathway genes, combining genes from different pathways to form a new pathway and by fusing segments from preexisting catabolic genes into new catabolic gene. A number of catabolic plasmids are listed in Table 9.10.

The conjugative plasmids are important agents of genetic exchanges for enhancing metabolic potential. Plasmids can be pooled in a single organism which may undergo recombination to evolve new pathways. For example, five different pathways are involved in aerobic degradation of toluene. Pathway encoded by

Table 9.10 Catabolic plasmids of *Pseudomonas* and related species

Bacterium	Plasmid	Size (kb)	Substrate
<i>P. putida</i>	pCAM		Camphor (moth repellent and used in plastics, varnishes, etc.)
<i>P. putida</i>	pSAL	85	Salicylate (substrate for aspirin manufacturing)
<i>P. putida</i> G-7	pNAH7	83	Naphthalene and salicylate
<i>P. putida</i>	pWW60-1	87	Naphthalene (insecticide, manufacture of indigo blue)
<i>P. arvilla</i>	pTOL	117	Toluene (component of gasoline for dyes and explosives)
<i>P. putida</i>	pWW174	200	Benzene (component of gasoline)
<i>P. putida</i>	pOCT		Octane (component of crude oil)
<i>P. putida</i>	pUC18		Dibenzothiophene naphthalene, phenanthrene
<i>P. conveca</i>	pNIC		Nicotine/nicotinic acid (tobacco industry waste)
<i>P. putida</i>	pRE4 pRE1	105	Isopropyl benzene (solvent)
<i>Pseudomonas</i> sp.	pCITI PEST100	100 544	Aniline Phenol
<i>P. putida</i> <i>Pseudomonas</i> sp.	pV1 150 pND50 pWR1	Mega	Phenol, cresol, 3,4-dimethyl phenol <i>p</i> -cresol 3-CBA
<i>P. putida</i>	pAC25 pAC31	117 108	3-CBA 2,5-DCBA
<i>P. diminuta</i>	pCS1		Parathion (insecticide organophosphate)
<i>Burkholderia cepacia</i>	p 2,4,5 -T	Mega plasmid	Herbicide
<i>Cupriavidus necator</i> JMB134	pJP4	77	3CBA, 2,4-D (herbicide)
<i>C. necator</i>	pJP1 pMAB1 pRC10	– 90 45	2,4-D 2,4-D 1,2,4-trichlorobenzene
<i>P. putida</i> NCI139816	pDTG1		Naphthalene
<i>P. fluorescens</i>	pLP6a	pLP6a	Naphthalene Anthracene Phenanthrene

TOL plasmid (pWWO) of *P. putida* PaW1 is a well-characterized pathway (Worsey and Williams 1975). Toluene is converted into benzyl alcohol, benzaldehyde, benzoate, and catechol which is further metabolized by meta-cleavage pathway by catechol 2,3-dioxygenase. *Pseudomonas putida* F₁ metabolizes toluene to *cis*-toluene dihydrodiol leading to methylcatechol which subsequently undergoes meta-cleavage. Three other pathways for toluene metabolism are operative in *P. cepacia* G-4 (*B. cepacia* G4) (Shields et al. 1989), *P. pickettii* PK01, and *P. mendocina* KP1

(Whited and Gibson 1991), and toluene is converted into ortho-, meta-, and para-cresol, respectively. The ortho- and meta-cresols result in methyl catechol while the *p*-cresol leads to protocatechuate formation. The peripheral oxygenases of the five pathways are specific. Xylenes are degraded by the *xyl* pathway encoded by TOL plasmid.

NAH and TOL plasmids in *Pseudomonas* are well-elucidated plasmids. A number of factors such as structure of genes, enzymes, substrate, and metabolites influence the expression of catabolic genes. The extensive catabolic capabilities of *Pseudomonas* are attributed to catabolic plasmids. The physical size of these plasmids enables them to encode large number of enzymes. A plasmid of 150 kb contains genes to encode nearly 150 enzymes. This provides for large regions of DNA to which no functions have been assigned. The TOL plasmid has 12 catabolic enzymes that catalyze cleavage of toluene and meta- and para-xylenes. The TOL plasmid is organized into three operons OP₁ and OP₂ and the regulatory region, *xylRS*. OP₁ codes for enzymes of upper pathway for degradation of toluene and xylene to benzoate and toluate comprising of genes *xylCAB*, whereas OP₂ comprises of genes encoding lower pathway, *xyl DLEGFJKIH* (Harayama et al. 1987). Two regulatory genes *xyl R* and *xyl S* regulate the genes of the upper pathway OP₁ (Pu) and lower pathway, respectively, OP₂ (Pm). The *xylR* protein binds the inducer xylene and activates the promoter of upper pathway and m-*xyl*-*xylR* complex then activates the OP₂(Pm). Several LysR-type transcriptional regulator proteins have been identified that are involved in the degradation of pathways such as benzoate, phenols, and chlorobenzoates. Other regulatory proteins of the ICIR family and AraC/XylS family play an important role in the regulation of aromatic catabolic pathways (Diaz and Prieto 2000; Tropel and van der Meer 2004).

Plasmids are also involved in the degradation of chlorinated compounds like 2CBA, 3CBA, chlorinated biphenyls (PCB) chlorophenols, and 2,4-D. The genes coding for peripheral pathways are generally encoded on plasmids in *Pseudomonas*, but in some strains, these gene sequences are located on chromosome, e.g., genes coding for toluene/benzene degradation in *P. putida* F₁ or genes coding for biphenyl degradation in *P. putida* F₁ or genes coding for biphenyl degradation in *P. pseudoalcaligenes* KF707 or may be located on nonconjugative plasmid. Such strains are used as acceptors for additional DNA. Besides the mediation of conjugative plasmids through conjugation process, the mobility of plasmids may be mediated due to association of IS elements and transposons (Tn). IS elements or transposons can even capture and affect mobilization of other genes as these can transpose within a replicon or between the replicons.

Genetic manipulation offers ways to engineer microorganisms with improved degradation pathways, expanded substrate specificity, and additional in situ pathways to develop “superbugs” that can efficiently scavenge environmental pollutants (Dwyer and Timmis 1990; Kocher and Kahlon 2003). The strategies may include designing of appropriately regulated gene expression circuits and improved protein/enzyme stability, catalytic activity, substrate affinity, and substrate specificity of the encoded catabolic enzymes and genetic assembly of

pathway modules to combine metabolic routes to create novel catabolic pathways (Fetzner 1998). *Pseudomonas* offer a particular advantage because of the presence of relatively nonspecific catabolic enzymes clustered on plasmids/chromosomes, divergence/convergence of peripheral metabolism with multiple forms of peripheral enzymes, effective transporter proteins, and solvent tolerant systems in *Pseudomonas* for combining pathways and selecting strains with increased adaptability and biodegrading ability. The gene organization and sequence similarity (~90 %) of the upper pathway was observed and the genes are arranged in the order *nahAaAbA-cAdBFCQED* representing different genes encoding enzymes of naphthalene pathway (Takizawa et al. 1994; Bosch et al. 1999a, b). Lower pathway-encoding salicylate hydroxylase (*nahG*), chloroplast ferredoxin-like protein (*nahT*), catechol 2,3-dioxygenase (*nahH*), hydroxymuconic semialdehyde dehydrogenase (*nahI*), hydroxymuconosemialdehyde hydrolase (*nahN*), 2-oxopent-4-enoate hydratase (*nahL*), acetaldehyde dehydrogenase (*nahO*), 2-oxocrotonate decarboxylase (*nahK*), and 4-oxalocrotonate isomerase (*nahJ*) have been sequenced in *P. stutzeri* AN10 and *P. putida* G7 and NC IB9816 and are arranged as *nahGTHINLOMKJ* (Bosch et al. 2000).

NAH (NAH7) is an 83 kb transmissible plasmid coding for naphthalene, first described in *P. putida* strain G7 (Dunn and Gunsalus 1973). Catabolic genes are organized in three operons—encoding upper pathway enzymes, the lower pathway enzymes catalyzing conversion of salicylate to TCA cycle intermediates by meta-ring-cleavage pathway, and encoding the regulatory protein (NahR). Both upper and lower pathway operons are regulated by *trans*-acting positive control regulatory protein, NahR (Yen and Gunsalus 1985). The *nah R* gene lies between the two operons and is required for high-level expression of the *nah* genes as well as their induction by salicylate.

Naphthalene catabolic plasmids (NAH plasmids) that have been isolated from a number of *Pseudomonas* strains, e.g., pWW60 of *P. putida* NC1B9816 and pKA1 from *P. fluorescens* 5R, were found to be similar to NAH7 of *P. putida* G7. Genes of the upper pathways have been sequenced from several strains with similar functions (Habe and Omori 2003).

9.4.5.2 Engineering Degradative Pathways

Engineering strains with better derivative capabilities was initiated by pooling plasmids from different strains to develop a superbug for simultaneous degradation of camphor, toluene, xylene, salicylate, naphthalene, etc. The organism was patented in the USA as the first living organism to be patented (Chakrabarty 1981). With the development of techniques of genetic manipulation, the strategy has undergone a change and is more precise and specific that allows gene amplification or modification of the specific gene of the pathway, optimization of gene function, and recruitment of heterologous genes. Various genetic approaches have been used to optimize enzymes and pathways for biodegradation (Cases and de Lorenzo 2005a, b).

The subject has been receiving a lot of attention and reviewed by Pieper and Reineke (2000), Saini and Kahlon (1998) and Menn et al. (2010). Some strains lack

the ability for complete degradation of a substrate due to the absence of a particular enzyme or they accumulate a dead-end product that may be toxic and is not further metabolized such as 3-chlorocatechol, or 4-chlorophenol. *Pseudomonas* sp. B13 was the first to be modified; the parent strain metabolizes 3-CBA to 3-chlorocatechol followed by ortho-cleavage. However, this strain did not utilize 4-methyl benzoate (4-MB) and 4-chlorobenzoic acid (4CBA). First benzoate 1,2-dioxygenase was replaced with its broad specificity counterpart from TOL plasmid pWWO (*Pseudomonas* sp. B13 FR1) enabling it to utilize 4-methyl benzoate (4 MB) and 4-CBA. However, *Pseudomonas* sp. B13 FR1 when grown on 4 MB, accumulated 4-methyl-2-ene-lactone as a metabolite as it lacked the specific isomerase. The gene for isomerase enzyme from *Alcaligenes eutrophus* was cloned in a cosmid vector pLAFR3 of *E. coli* and transferred to *Pseudomonas* sp. B13 FR1 by conjugation (Rojo et al. 1987; Reineke 1998; Wittich and Wolff 2007). The resulting exconjugant *Pseudomonas* sp. B13 FR1 (pFRSC20P) utilized 4 MB, 4 CBA, and 3 CBA as growth substrates. Several other examples are available where degradative genes have been pooled. A derivative of 2,4,5-T degrading *Pseudomonas cepacia* AC1100 was constructed with the ability to degrade 2,4-D as well as 2,4,5-T by transferring PJP₄ from *Alcaligenes eutrophus* JMP134, a 2,4-D degrader. The new strain RHJ1 effectively degrades both 2,4-D and 2,4,5-T, while the parent strains were specific for 2,4-D or 2,4,5-T only (Haugland et al. 1990; Perez-Pantoza et al. 2008). In our laboratory, the 3-CBA plasmid DNA purified from *P. putida* DP4 was transferred to *E. coli* DH5 by CaCl₂ induced transformation. The plasmid genes were successfully expressed in *E. coli* DP5 as the transformants were selected on 3CBA medium (Saini and Kahlon 1998). Wu et al. (2006) reported the cloning and expression of genes coding for partial reductive pathway for 4-chloronitrobenzoate and nitrobenzene from *Comamonas* sp. strain CNB1. A hybrid strain, *P. putida* TB105 was constructed by cloning the genes encoding TOD pathway on broad host multicopy vector RSF1010 and introducing into the TOL strain *P. putida* mt-2. The hybrid pathway channeled the dihydrodiols from benzene, toluene, and *p*-xylene in TOD pathway into TOL pathway for complete mineralization (Lee et al. 2006).

Genetic engineering has been extensively used to evolve strains (Table 9.11) with higher enzyme stability and specificity, new metabolic pathways and regulatory control mechanisms, introduction of marker gene for monitoring the recombinant organism in the environment, and development of biosensors for detection of pollutants in the environment (Davison 2005; Sayler and Ripp 2000).

9.4.5.3 Enzyme Engineering

In contrast to genetically modified organisms, the key enzyme can be modified to enhance their stability, specificity, and affinity for the substrate with a view to enhance the overall process. Dioxygenases and dehalogenases are the key enzymes to attack the pollutants. The enzymes have very specific amino acid sequence in the active center and its tertiary structure. Modifications are introduced by site-directed mutagenesis or by predicting the model structure by introducing alterations in amino acid sequence, and specific recombinant gene is synthesized with altered

Table 9.11 Genetically engineered microorganisms for degrading organic compounds (Wasilkowski et al. 2012)

GEM	Gene introduce	Compound degraded	References
<i>P. putida</i> KT2442 (PNF142::TnMod.OTc)	PNF142 plasmid <i>gfp</i>	Naphthalene	Filonov et al. (2005)
<i>P. putida</i> PaW85	PWWO plasmid	Petroleum	Jussila et al. (2007)
<i>P. putida</i> PaW340 (pDH5)	pDH5 plasmid	4-CBA (stable intermediate of PCB and DDT degradation)	Massa et al. (2009)
<i>P. fluorescens</i> HK44	Lux CDABE	Naphthalene	Sayler and Ripp (2000)
<i>P. fluorescens</i> F113 rif ^r pcbrn BP1:: <i>gfp</i> mut 3	Operon <i>bph gfp</i>	Chlorinated biphenyls	Boldt et al. (2004)
<i>Burkholderia cepacia</i> L. S.2.4	pTOD plasmid	Toluene	Barac et al. (2004)
<i>B. cepacia</i> VM1468	pTOM-Bu61 plasmid	Toluene	Filonov et al. (2005)
<i>Comamonas testosteroni</i> SB3	PNB2:: <i>dsRed</i> plasmid	3-chloro-aniline	Bathe et al. (2009)
<i>Escherichia coli</i> Atz 3	Atrazine chlorohydrolase	Atrazine	Strong et al. (2000)

sequence and cloned in the appropriate host for synthesis of the enzyme with predicted properties. Enzymes such as cytochrome P₄₅₀CAM catalyzing camphor oxidation, the biphenyl dioxygenase of *Pseudomonas* LB400 and *P. pseudoalcaligenes* KF707, and toluene and naphthalene dioxygenases have been manipulated. A site-directed mutagenesis of four nucleotides led to a change of four amino acids in dioxygenase reductase component coded by *bphA* gene. The modified novel enzyme combined the broad substrate specificity of *Pseudomonas* sp. LB400 and efficiency of homologous enzyme from *P. pseudoalcaligenes* KF707 to degrade di-, tri-, and tetra- para-substituted PCBs (Alcalde et al. 2006; Rao et al. 2010).

Recent emphasis in bioremediation has been the utilization of science of omics including the genomics, proteomics, transcriptomics, and metabolomics with the aim to model enzymes and strains that are more versatile and able to withstand the stresses of the natural environment (Thomas et al. 2008). Genetic modification of indigenous microflora would provide a better alternative as they can easily adjust in the environment. However, the big issue is the spread of genetically modified organisms and their consequences due to the persistence of naturally undesirable genes in the environment and their transfer to the indigenous species (Diaz 2004; Zhao and Poh 2008). Thus the risk assessment has become an important area of research dealing with issues of survival, gene transfer, containment, and ecological impact of GMO released in the environment. The risk can be minimized by biological containment system. For example, the promoter may be so designed that it functions in the presence of a specific pollutant, and as the level of pollutant

decreases, it is repressed and the promoter controlling suicide is induced (Contreras et al. 1991). Another system is nucleases containing a killing system that will hydrolyze DNA also so that it is not transferred to other organisms from the dead ones (Ahrenholtz et al. 1994).

Only one organism, *Pseudomonas fluorescens* HK44, has been allowed for controlled release for field application. *Pseudomonas fluorescens* HK44 possesses a NAH plasmid (pUTK21) which has got within its promoter a transposon based *lux* (bioluminescence) gene insertion for monitoring biodegradation, and control (Sayler and Ripp 2000). *Pseudomonas fluorescens* HK44 degrades naphthalene and simultaneously produces a bioluminescent signal for monitoring. *Pseudomonas fluorescens* HK44, ($\text{nah}^+ \text{sal}^+ \text{lux}^+$) derived from Pf5R ($\text{nah}^+ \text{sal}^+$)-containing plasmid pKA1 mutagenized by *lux* CDABE-containing transposon, Tn4431 on suicide vector plasmid pUCD623i. Both Pf5R and RfHK44 have genes for the upper (*nah* ABCDEF) and lower (*nah* GHIJK) salicylate, pathways of naphthalene degradation. Thus strain HK44 serves as a reporter for naphthalene bioavailability and biodegradation and, through bioluminescence signaling can be used as a tool for monitoring bioremediation processes.

Pseudomonas aeruginosa (NRRL B-5477) and *P. putida* NRRL B5473 were patented. They contained genes for naphthalene, salicylate, and camphor degradation (<http://www.freebase.com>)

Pseudomonas species figure prominently to unravel as to how microbes recycle organic molecules in the environment. Some of the novel reactions have been harnessed for synthesis of specialty chemicals. *Pseudomonas putida* strains catabolize natural products such as vanillin, α -pinene, limonene, mandelate, camphor, and adamantanone and industrial compounds such as styrene, bromoxynil, methyl *tert*-butyl ether (MTBE), and trichloroethylene. The degradation products of these are funnels to diols such as catechol, protocatechuate, and hydroxybenzoate which are further metabolized to yield products that enter the TCA cycle and other central catabolic pathways.

On the basis of genome sequence analysis, *P. putida*, *P. fluorescens* Pf0.01, *P. fluorescens* SBW25, *Burkholderia cepacia* J2315, *Sphingomonas aromaticivorans* F199, *Rhodococcus* sp. 124, and *Rhodococcus* sp. RHA1 have been regarded as critical for recycling of organic carbon on the planet. Detailed genomic and metabolic analysis will help us design herbicides and pesticides with a high degree of degradability in the environment and the pathways for their degradation can be predicted (Wackett 1996).

Genome sequence analysis and in silico modeling of metabolic potential offers a great advantage for construction of strains specific for certain metabolic functions using system's biology techniques. Nogales et al. (2008) presented the metabolic reconstruction of *P. putida* KT2440::iJN746 which carries as many as 746 genes representing 950 biochemical reactions and 911 metabolites. iJN746 captures catabolic pathways for toluene, benzoate, phenylacetate, and nicotinate degradation and polyhydroxyalkanoate (bioplastics) biosynthesis.

9.5 Degradation of Pesticides

Chemical pesticides are extensively used in agriculture as insecticides, herbicides, fungicides, etc. to control insect pests, weeds, and plant diseases, etc. as well as public health measures to control vector-borne diseases (Agrawal et al. 2010; Damalas 2009). Use of pesticides began with the introduction of DDT (2,2 bis (*p*-chlorophenyl)-1,1,1-trichloroethane) in the 1940s as an effective plant protection measure in agriculture as well as in public health to control mosquito carrier of malarial parasite (Turusov et al. 2002). Subsequently other chlorinated pesticides, viz., aldrin, HCH (BHC), chlorodane, and mirex, were introduced. Later other groups of pesticides such as organophosphates, carbamates, and pyrethroids were introduced. Important features of these pesticides are listed in Table 9.12.

These compounds became very popular as these were relatively cheap, very effective as insecticides, and were easy to use. At the same time not much emphasis was laid on the fact that they will affect the nontarget organisms as well as their behavior in the environment particularly soil and water which receive roughly about 95 % of herbicide and 98 % of sprayed pesticide because only a fraction of the pesticide is utilized by the target flora and fauna. It was 20 years later in the 1960s when the reports about accumulation of DDT and other organochlorinated compounds started appearing as these compounds and their metabolites were resistant to degradation and persisted in the environment. DDT and its isomers disrupted the endocrine system, resulting in impaired reproduction of wildlife species. DDT, DDD, and DDE are potential carcinogens and the use of DDT in the USA was banned in the 1970s except for emergency use for control of vector-borne diseases (Spencer et al. 1996). In India also organochlorinated pesticides, DDT, aldrin, and HCH were banned for agriculture except for the γ isomer of HCH

Table 9.12 General characteristics of major groups of chemical pesticides

Pesticide	General characteristic
Organochlorines: DDT, aldrin, lindane, chlorodane, mirex	Comprised of C, H, Cl atoms, rarely oxygen. Nonpolar, soluble in lipids, accumulate in fatty tissue, transferred through food chain, toxic to animals, and highly persistent
Organophosphates: malathion, methyl parathion, diazinon	Possess phosphorus atom in the center, more stable but less toxic in the environment, less persistent than organochlorines, soluble in water and organic solvents, absorbed by plants, and transferred to leaves and stems. Suitable for leaf eating insects. Mode of action—affect the central nervous system
Carbamates: Sevin, carbaryl	Based on plant alkaloid <i>Physostigma venenosum</i> . Effective against a limited array of insects but highly toxic to vertebrates. Low persistence
Pyrethrins and pyrethroids	Similar to pyrethrins, i.e., alkaloids obtained from petals of <i>Chrysanthemum cinerariaefolium</i> . Several of similar compounds synthesized and are referred as pyrethroids. Low in persistence and safest to use. Used as household insecticides

(lindane) as it was quite effective and was not as problematic as α -, β -, and δ isomers. Heavily contaminated sites with HCH have been identified all over the world. Residues of HCH have been reported in air, soil, water, food, milk, fish, mammals, and human blood and adipose tissue (Lal et al. 2010; Rangachary et al. 2012).

Pesticides have become an integral part of agriculture. Without pesticides the high losses to horticulture, vegetable, and cereal crops are estimated at 78 %, 54 % and 32 %, respectively. Europe is the largest consumer of pesticides with Asia being the next and major producers are China, the USA, France, Brazil, and Japan. Because of the environmental impact of chemical pesticides, emphasis has been on development of biological pesticides (Zhang et al. 2011). However, the unregulated and indiscriminate use of pesticides has been a cause of concern because of adverse effects on human health, other forms of life, and ecosystems depending upon the degree of sensitivity of the organism and toxicity of the pesticide. The continued use of pesticides has resulted in contamination of ground and underground waters and soil and even enters the food chain. Over the years several species of birds feeding on insects and grains have become extinct and many insect species have developed resistance to insecticides. Thus their impact on human health and the environment is important because of their persistence in the environment, bioaccumulation and bioconcentration, and toxicity against human beings and other nontarget organisms (Damalas 2009; Lamberth et al. 2013).

In natural environment, the fate of pesticide is determined by physicochemical and biological factors. On entry into soil the sorption of the pesticide on the soil particle takes place which control their transfer and bioavailability. The behavior of pesticides in soil, efficacy as a pesticide, persistence, and potential as soil contaminant is determined by their retention and degradation on the soil particle (Gevao et al. 2000). Various biotic and abiotic processes by which the pesticide undergoes transformation have been highlighted recently (Ortiz-Hernandez et al. 2013; Lal et al. 2010).

To overcome the problem of environmental contamination by prevalent organic pollutants, bioremediation has emerged as an important technique as it does not suffer from inherent limitations of the conventional methods and is cost-effective. Bioremediation involves degradation of chemical pollutants under the natural environment or in isolation under natural or with suitable modification with a view to enhance the process of degradation of naturally occurring or laboratory cultures or even genetically modified microorganisms.

Soil microflora play an important role in biotransformation of pesticides. On regular exposure soil bacteria develop ability to degrade pesticides as these can serve as sources of carbon and energy. Among these, members of genus *Pseudomonas* and many other proteobacteria are prominent for aerobic degradation. Besides the use of pesticides in agriculture, soil also gets contaminated with other chemicals, industrial effluent, sewage sludge, activated sludge, wastewaters, etc. Isolation and characterization of microorganisms from the contaminated and modern analytical and molecular techniques have enriched our knowledge of the

Table 9.13 Important strains of *Pseudomonas* and related aerobic bacteria with ability to degrade pesticides

Compounds	<i>Pseudomonas</i> strain
Organochlorinated DDT	<i>P. acidovorans</i> , <i>P. aeruginosa</i> 640X <i>P. aeruginosa</i> B5816 pNAH + SAL _O <i>P. aeruginosa</i> B5827 pNAH + SAL _m <i>Pseudomonas</i> sp. 27, <i>Cupriavidus necator</i>
Lindane (γ HCH)	<i>Pseudomonas aeruginosa</i> , <i>P. putida</i> <i>Pseudomonas</i> sp., <i>Sphingobium japonicum</i> <i>S. indicum</i> B90A, <i>S. francense</i> sp. ⁺ <i>Sphingobium</i> sp., <i>Sphingomonas</i> sp.
Aldrin	<i>Pseudomonas</i> sp. 94, <i>Pseudomonas</i> sp. 138
Carbamates carbofuran	<i>Pseudomonas</i> sp., <i>Flavobacterium</i> sp. <i>Arthrobacter</i>
Triazines-atrazine	<i>Pseudomonas</i> sp., <i>Pseudomonas</i> sp. <i>Pseudomonas</i> sp. ADP
Phenoxy compounds	
2,4-D	<i>C. necator</i> , <i>B. cepacia</i>
Dicamba	<i>P. maltophilia</i>

mechanisms (how), occurrence (what), and identity (who) of the microorganisms that are effective in biodegradation of organic environment pollutants.

Because of their diversity and metabolic versatility, members of genus *Pseudomonas* readily adapt and have been recognized as an efficient biological system for degradation of toxic chemicals (Table 9.13).

9.5.1 Organochlorinated Insecticides

Degradation of DDT (2,2-bis(*p*-chlorophenyl) 1,1,1-trichloroethane) proceeds through reductive dechlorination to give rise to DDD or DDE. The technical grade DDT comprises of 14 different chemicals containing 65–80 % active ingredient *p,p'*-DDT, 15–21 % inactive *o,p*-DDT, 4 % DDD, and up to 1.5 % of 1-LP-chlorophenyl 2,2,2-trichloroethanol. Normally DDD is formed from DDE, but aerobic bacteria like *B. megaterium* convert DDT into DDD without the formation of DDE. *E. coli* under aerobic conditions converted DDT to DDD (75 %) and DDE (25 %). Dieldrin-degrading *Pseudomonas* sp. degraded DDT to DDD. DDT is highly persistent and has a long life of 2.5–17 years. Under aerobic conditions, it undergoes only cometabolism with other substrates as carbon and energy sources. Under natural conditions several metabolites have been identified which are produced as dead-end products. A number of dehydrohalogenases, dehalogenases, dioxygenases, hydrolases, and dehydrogenases have been identified for conversion of DDT into 4-chlorobenzoic acid as the key intermediate (Reiner et al. 1989).

Pseudomonas aeruginosa 640x isolated from DDT-contaminated soil harbors a plasmid coding for ortho-cleavage (strain BS816) and converts 89 % DDT into

DDD, DDDE (1,1,-dichloro-2,2-bis(*p*-chlorophenyl) ethylene), PPA (phenyl propionic acid), and PAA (phenylacetic acid). Another strain of *Pseudomonas* sp. has been identified with the ability to use diphenylethane as sole source of carbon and energy. Diphenylethane is converted into 2-phenyl propionic acid and metabolized further. DDT also undergoes cometabolism with readily utilizable substrates as primary substrates (Aislabie et al. 1997; Thomas et al. 2008).

Lindane, the γ -isomer of hexachlorocyclohexane which is extracted and purified from technical HCH, is used as an insecticide. All other isomers, α -, β -, and δ are banned as they are more toxic and problematic from the point of view of the environment. Residues of HCH isomers in air, water, soil, food, and milk have been reported from several countries and heavily contaminated sites have also been identified in several countries. The ϵ -isomer is relatively unstable while other four isomers undergo bacterial degradation both anaerobically and aerobically. Successive dichloroelimination and then dehydrochlorination are the important steps for formation of chlorobenzenes, which is degraded by peripheral catabolic pathways.

Among the aerobic degrading bacteria, members of family *Sphingomonadaceae* of class β -proteobacteria are predominant, though some of the important species such as *Sphingobium japonicum*, *S. indicum* B90A, and *S. francense* sp⁺ were earlier classified as *Sphingomonas paucimobilis* (*Pseudomonas paucimobilis* strain SS86). Besides these, *P. aeruginosa*, *P. aeruginosa* ITR5, *P. putida*, and *Pseudomonas* sp. have been reported to degrade lindane aerobically. *Pseudomonas aeruginosa* ITRC-5 degraded 98 % of γ -HCL after 15 days of incubation at 15 % water content, pH 8.0, and temperature 28 °C, with inoculums of 10⁶ cells/g soil. Chaudhary et al. (2006) reported > 80 % degradation of γ -HCH by *P. aeruginosa* ITRC-5 in 24 h of incubation. A consortium of 10 bacterial species comprising of one each of *Flavobacterium* sp., *Vibrio* sp., *Burkholderia* sp., and *Pseudomonas* sp. was able to degrade 90 % of γ -HCH in 72 h of incubation (Afsar et al. 2005).

Lindane degradation has been a subject of extensive studies. The genes and enzymes for degradation of γ -lindane have been characterized in *S. paucimobilis* UT26. Six enzymes are involved in the conversion of γ -HCH to β -ketoadipate; the enzymes are dehydrochlorinase (*linA*), halohydrolase (*linB*), dehydrogenase (*linC*), reductive dechlorinase (*linD*), ring cleavage dioxygenase (*LinE*), and reductase (*linF*) (Nagata et al. 1999, 2007; Endo et al. 2005). The genes for these are highly conserved, have been characterized, and partially or completely sequenced (Kumari et al. 2002; Lal et al. 2006, 2010). The genes are regulated by LysR family transcriptional regulation. Other enzymes involved in lindane degradation are—maleylacetate reductase (*linF*) ring cleavage oxygenase (*linEb*), acyl-CoA transferase (*linG*), acyl-CoA transferase (*linH*), ICLR family transcriptional regulator (*linI*), thiolase (*linJ*), putative ABC transporter system, inner membrane protein (*linK*), putative ABC transporter ATPase (*linL*), putative ABC transporter system, periplasmic protein (*linM*), putative ABC transporter system, lipoprotein (*linN*), and dehydrogenase (*linX*). The sequence of reactions for aerobic metabolism is presented in Fig. 9.8

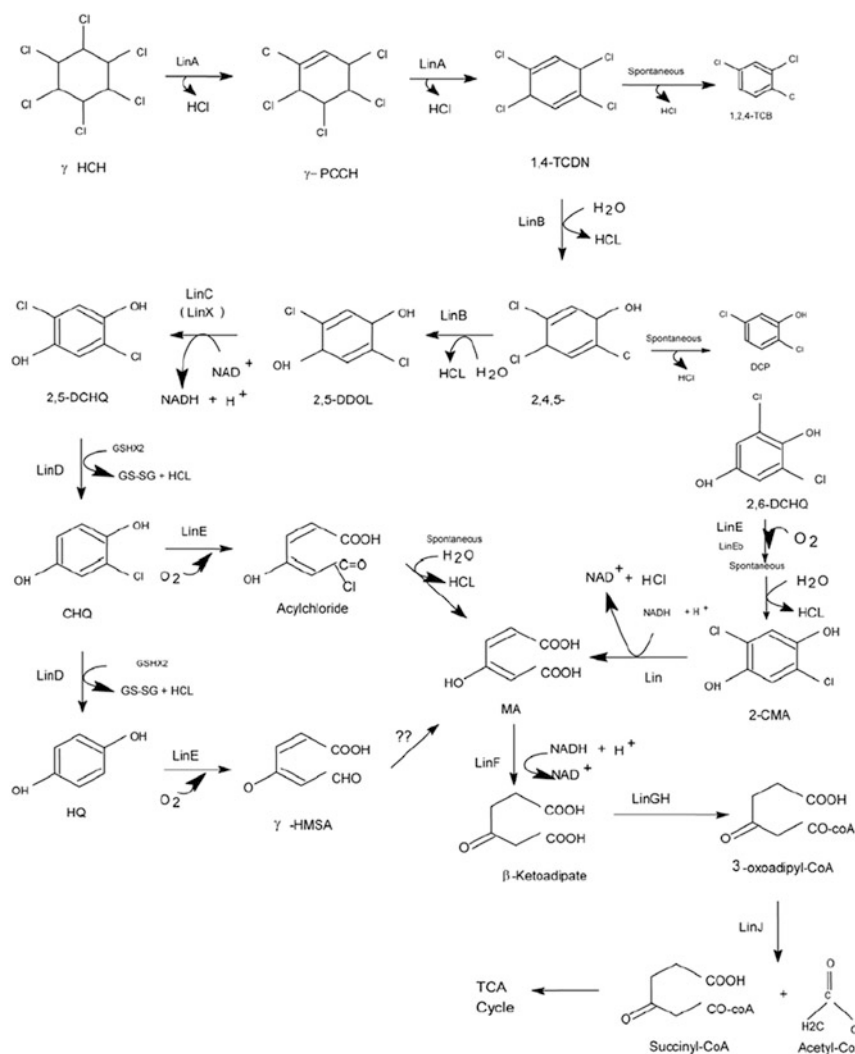


Fig. 9.8 γ -HCH degradation pathway in *Sphingobium japonicum* UT26 (Nagata et al. 2007). γ -HCH, γ -hexachlorocyclohexane; γ -PCCH, gamma-pentachlorocyclohexene; 1,4-TCND, 1,3,4,6-tetrachloro-1,4-cyclohexadiene; 1,2,4-TCB, 1,2,4 trichlorobenzene; 2,4,5-DNOL, 2,4,5-trichloro-2,5-cyclohexadiene-1-ol; 2,5-DCP, 2,5-dichlorophenol; 2,5-DDOL, 2,5-dichloro-2,5-cyclohexadiene-1,4-diol; 2,5-DCHQ, 2,5-dichlorohydroquinone; 2-CHQ, 2-chlorohydroquinone; HQ, hydroquinone; γ -HMSA, gamma-hydroxymuconicsemialdehyde; MA, maleylacetate; 2-CMA, 2-chloromaleylacetate; and TCA, tricarboxylic acid cycle

Aerobic degradative pathway has proved very useful as a model for investigating fundamental issues in microbial and molecular evolution. It appears that bacteria acquire the requisite capabilities by assembling genes from different sources.

9.5.2 Organophosphates

Organophosphorus compounds are widely used as insecticides accounting for about 1/3 of the total insecticide sales. They are also used as plasticizers, air fuel ingredients, and chemical warfare agents (Singh and Walker 2006). They have high mammalian toxicity but have the advantage that these are readily degraded in the natural environment as they are biodegradable and have short half-life. *Flavobacterium* sp. was the first bacterial strain to be identified to degrade organophosphates. Later several genera have been characterized to degrade organophosphate pesticides in the natural environment. Members of genus *Pseudomonas* also metabolize organophosphates either as cometabolism at the expense of some other compound as source of carbon and energy or they utilize these as sources of carbon or carbon and nitrogen (Yali et al. 2002). The strains of *Pseudomonas diminuta*, *P. stutzeri*, *Pseudomonas* sp., *P. putida*, *Pseudomonas* sp. A₃, *Pseudomonas* sp. WBC, *Pseudomonas* sp., *P. monteilii*, *P. mendocina*, *P. aeruginosa* F10B, and *Pseudomonas* spp. have been isolated and characterized to degrade chlorpyrifos, parathion, methyl parathion, glyphosate, coumaphos, monocrotophos, fenitrothion, and diazinon (Singh and Walker 2006; Singh and Singh 2003; Bhadbhade et al. 2002). Most of these were introduced as insecticides during 1965–1971. These contain phosphorus as a phosphate ester or as a phosphonate. Bacterial degradation proceeds through hydrolysis of *P-O*-alkyl and *P-O*-aryl bonds as a significant step in detoxification.

Hydrolysis of organophosphorus results in reduction of toxicity to a large extent. Chlorpyrifos is extensively used as an insecticide and is effective against a broad spectrum of insect pests of agricultural crops. Its degradation involves both chemical hydrolysis and microbial activity with the formation of 3,5,6-trichloro-2-pyridinol (TCP) as the major degradation product. TCP has antimicrobial properties and prevents proliferation of chlorpyrifos-degrading microorganisms. The degradation of chlorpyrifos is slow and reported to be resistant to enhanced degradation (Singh et al. 2008).

Both *Flavobacterium* sp. and *P. diminuta* cometabolize chlorpyrifos (Sethunathan and Yoshida 1973; Serdar et al. 1982). *Enterobacter* isolated from Australian soil has been reported to show enhanced degradation of chlorpyrifos to diethylthiophosphoric acid (DETP) and trichlorophenol (TCP) and utilizes DETP as source of carbon and phosphorus (Singh et al. 2003, 2004; Yang et al. 2006). *Pseudomonas acidovorans* utilizes DETP as sole source of sulfur.

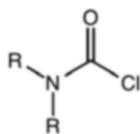
Only one bacterium, *Pseudomonas* sp., has been isolated which can mineralize (TCP) involving reductive dechlorination pathway to chlorodihydro-2-pyridone which is further dechlorinated into tetra-hydro-2-pyridone. Ring cleavage of this results in the formation of maleimide-semialdehyde which is metabolized to water, CO₂, and ammonium ions (Singh and Walker 2006).

Parathion (*O,O*-diethyl-*O-p*-nitrophenyl phosphorothioate) is one of the most toxic insecticides but is rapidly degraded in biologically active soil system. Several species including *Pseudomonas* degrade parathion. *Pseudomonas* and other aerobic bacteria hydrolyze parathion to *p*-nitrophenol and many pseudomonads like *P. putida* and *P. cepacia* can metabolize *p*-nitrophenol further to the intermediates

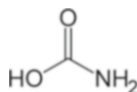
of TCA cycle (Carnett 2002; Cui et al. 2001). *Pseudomonas* sp. degrades *p*-nitrophenol to *p*-benzoquinone by using a monooxygenase (Singh and Walker 2006). *Pseudomonas putida* and *P. cepacia* further give rise to 1,2,4-benzenetriol which undergoes cleavage by benzene oxygenase resulting in the formation of maleylacetate, the product of which enters the TCA cycle (Singh and Walker 2006). *P. aeruginosa* utilize *p*-nitrophenol as a source of carbon energy and thus complements the pathway of *P. stutzeri* which cometabolically degrades parathion to *p*-nitrophenol and diethyl thiophosphate (Urlacher et al. 2004). Biodegradation of other organophosphates have been discussed in detail by Singh and Walker (2006), Singh et al. (2008) and Ortiz-Hernandez et al. (2013).

9.5.3 Carbamates

Carbamates are important as pesticides in agriculture because they have broad spectrum of activity, relatively degraded fast, and have low degree of toxicity toward human beings (Wolfe et al. 1978). Like organophosphates they are also neurotoxic and inhibit the hydrolysis reaction of acetylcholine (ACH). Carbamates are esters of carbamates and organic compounds derived from carbonic acid. These are divided into benzimidazole-, *N*-methyl, *N*-phenyl-, and thiocarbamates.



Carbamate acid



Carbamic acid

General structure of carbamates

A number of bacteria representing the genera *Pseudomonas*, *Flavobacterium*, *Achromobacterium*, *Sphingomonas*, and *Arthrobacter* have been isolated and characterized for carbofuran degradation in the environment. Carbofuran was degraded first to carbofuran phenol and then to 2-hydroxy-3-(3-methylpropan-2-ol) phenol by *Sphingomonas* (Kim et al. 2005). *Pseudomonas* sp. catabolizes fungicide and carbendazin to 2-aminobenzimidazole. In general the individual organism has a limited capacity to degrade xenobiotic pesticides and a consortium gives better results and brings about complete degradation. A strain of *P. aeruginosa* isolated from soil contaminated with carbofuran brings about complete degradation of carbofuran in minimal medium and uses it as source of carbon and energy (Naqvi et al. 2013).

Earlier three strains of *Pseudomonas* sp., C₄, C₅, and C₆ isolated from soil contaminated with carbaryl, have been reported to utilize carbaryl as source of carbon and energy. Carbaryl is oxygenated to 1-naphthol which is again oxygenated by a monooxygenase to give 1,2-dihydroxynaphthalene; cleavage results in salicylaldehyde and pyruvic acid. Salicylaldehyde is further oxidized to salicylic acid and gentisic acid which are metabolized resulting in products that enter the

TCA cycle. The key enzymes carbaryl hydrolase, 1-naphthol hydroxylase, 1,2-dihydroxynaphthalene dioxygenase, and gentisate dioxygenase are involved leading to the formation of maleylpyruvate.

Iyer et al. (2013) have reported the isolation and characterization of *Pseudomonas* sp. strain POXNO1 from soil very similar to *P. putida* S16, a nicotine degrading strain. Strain POXNO1 has genes for degrading paraoxon, the organophosphorus degrading (*opd*) gene identical to that of *Flavobacterium* sp. ATCC 27551 (*Sphingobium fuliginis*), and it can assimilate aromatic compounds phenol, benzene, and toluene based on gene cluster *pheARKLMNOPOQ* and benzoate on gene cluster *benRABCDKGEF*. This versatility in degradation of xenobiotics makes it suitable for multipurpose bioremediation. Genes for catabolic function of aromatics are similar to *Pseudomonas putida* S16.

9.5.4 Atrazine

Pseudomonas sp. strain ADP is the best characterized and model organism for degradation of atrazine, an herbicide of s-triazine group. *Pseudomonas* ADP brings about complete degradation of atrazine to CO_2 and NH_4^+ (Mandelbaum et al. 1995). This strain can tolerate and degrade up to 1000 mg l^{-1} of the herbicide. Three genes, *atzA*, *atzB*, and *atzC*, have been characterized in *Pseudomonas* sp. strain ADP and compared with five other atrazine-degrading bacteria (de Souza et al. 1998a). The rate of mineralization and proliferation of the catabolic microorganisms is correlated to the copy number of the genes. The absence of atrazine-dechlorinating gene, *atzA* limited degradation of atrazine. Degradative pathway of atrazine in *Pseudomonas* sp. ADP is hydrolytic rather than oxidative involving four steps: dehalogenation, N-dealkylation, deamination, and ring cleavage (de Souza et al. 1998c) (Fig. 9.9). The genes *atzABC* are located on a self-transmissible plasmid (de Souza et al. 1998b).

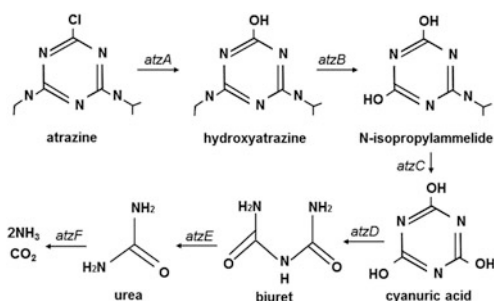


Fig. 9.9 Bacterial degradation of atrazine, the genes *atzA* (atratriazine chlorohydrolase), *atzB* (hydroxyl atrazine ethylaminohydrolase), *atzC* (*N*-isopropylammelide isopropylaminohydrolase), *atzD* (cyanuric acid aminohydrolase), *atzE* (biuret aminohydrolase), and *atzF* (allophanate hydrolase) code for the enzymes mentioned within parentheses. Gene *arzR* is LysR-type regulator

The genes *atzA*, *atzB*, and *atzC* encode atrazine chlorohydrolase (*AtzA*) and hydroxyatrazine ethylaminohydrolase (*AtzB*), and *N*-isopropylammelide isopropylaminohydrolase (*AtzC*) convert atrazine to cyanuric acid (de Souza et al. 1998a). These are located on the plasmid ~100 kb, pADP-1 (de Souza et al. 1998b). Complete sequence of pADP-1 has shown that genes *atzD*, *atzE*, and *atzF* for degradation of cyanuric acid to CO₂ and NH₃ are also located in a contiguous cluster on the plasmid ADP-1. The *atzD* gene encodes cyanuric acid aminohydrolase to form biuret, which is further hydrolyzed to urea and then to CO₂ and NH₃ by biuret hydrolase and urease (Devers et al. 2007).

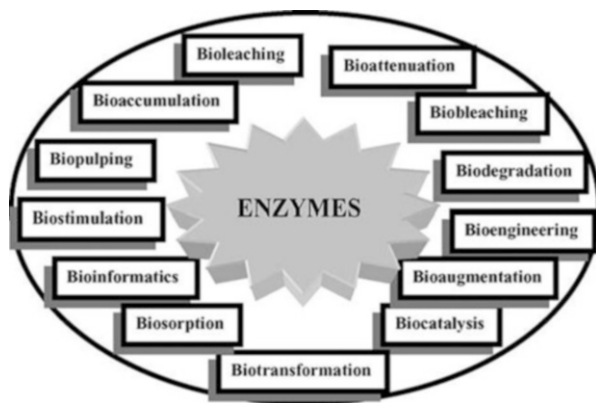
The genes *atzA*, *atzB*, *atzC* are expressed as constitutive genes and are not regulated by induction by atrazine or repression by other N-sources. However, genes *atzD*, *atzE*, and *atzF* are regulated by RpoN (σ54) and are induced by cyanuric acid and nitrogen depletion.

Pseudomonas sp. EGD-AKN5 has multiple degradative capacities for atrazine, benzoate, phenol, toluene, and catechol. Draft genome sequence has further demonstrated that all the genes *atzA/B/C/D/E/F* coding for degradation of atrazine via chlorohydrolase were active in this organism. Phenol was converted to catechol via multicomponent phenol hydroxylase of *phc* pathway. The organism has potential for exploitation for bioremediation by utilizing phenol as carbon source and atrazine as nitrogen source (Bhardwaj et al. 2015).

9.5.5 Enzyme and Genes in Pesticide Degradation

Enzymes are used for several processes as they have the advantage of specificity and also eliminate the risk of being spread into the environment (Fig. 9.10). Hydrolases are the major group of enzymes which catalyze hydrolysis of ester, peptide bond, carbon-halide bond, tri-esters, etc. Phosphotriesterases are involved in hydrolysis and detoxification of organophosphates (Baffi et al. 2008). Organophosphorus hydrolase (OPH encoded by *opd* gene), methyl parathion hydrolase (MPH encoded by *mpd* gene), and hydrolysis of coroxon (HOCA, encoded by *hoca*

Fig. 9.10 Overall view of the role of enzymes in the bioremediation processes (Whitley and Lee 2006)



gene) have been identified in *Pseudomonas diminuta* MG and *P. monteilii* (Latifi et al. 2012; Ortiz-Hernandez et al. 2013). Many pesticides like organophosphates, carbamates, and pyrethroids have carboxylic esters which are hydrolyzed by esterase. These are highly variable and are the most important enzymes in metabolism of xenobiotic compounds (Bansal 2012). Oxidoreductases are a broad group of enzymes that catalyze transfer of electrons from one molecule to another. Many of these enzymes require additional cofactors either as electron donors or electron acceptors or both. These enzymes are useful in bioremediation and catalyze oxidation-reduction reactions using O_2 as electron acceptors. Glyphosate oxidoreductase (GOX) catalyzes glyphosate degradation by *Pseudomonas* sp. LBr. This group of enzymes is involved in a large number of reactions in central and peripheral metabolism of xenobiotic compounds such as endosulfan, malathion, catechol, and other diols. *Pseudomonas aeruginosa* and *Burkholderia cepacia* hydrolyze endosulfan to yield less toxic metabolite endosulfan diol (Rodrigues et al. 2006; Senthilkumar et al. 2011).

Mixed function oxidases (MFO; EC1.14.14.1) are a group of enzymes in which an atom of molecule of oxygen is incorporated into the substrate, while the other is reduced to water. The reaction requires NADPH as a coenzyme and oxygen. This enzyme system comprises of two enzymes, cytochrome P450 and NADPH-cytochrome P450 reductase, the membrane proteins. They are referred to as cytochrome P450 monooxygenases and are active in the metabolism of a wide variety of xenobiotics (Urlacher et al. 2004; Weir et al. 2006).

Cytochrome P450 is a large family of well-characterized monooxygenases that also have potential in industrial processes. Cytochrome P450 enzymes have broad substrate range and even catalyze biochemically calcitrant reactions such as oxidation or hydroxylation of nonactivated carbon atoms and are suitable for remediation of environmentally persistent pesticide residues. Cytochrome P450 enzymes are widely distributed in prokaryotes and eukaryotes and contain iron-containing porphyrin group that absorbs at 450 nm upon binding to carbon monoxide. MFOs metabolize a wide range of compounds such as organophosphates, carbamates, pyrethroids, DDT, etc. (Alzahrani 2009).

Dioxygenases incorporate both atoms of oxygen molecule to form a diol which is subsequently cleaved to form a linear molecule from an aromatic ring. They are very important in metabolism of aromatics and xenobiotics. Dioxygenase, TOD, has been characterized from *P. putida* for degradation of the herbicide trifluralin. Haloalkane dehalogenase are very important in dechlorination of chlorinated pesticides and chemical pollutants. Dehalogenases (*linB*) have been identified in *Sphingobium* sp. and *Sphingomonas* sp. degrading β and δ isomers of HCH and *linA* in *Sphingobium* sp. and *Sphingomonas* sp. for γ -HCH degradation. Dechlorinases, *atzA*, and DMO have been characterized in *Pseudomonas* sp. ADP and *P. maltophilia*, respectively, for degradation of atrazine and dicamba (Scott et al. 2008).

The genetic basis of degradation of pesticides has been investigated in some strains of genus *Pseudomonas*, *Ralstonia*, *Sphingomonas*, and *Burkholderia*, etc. Most of the genes for catabolic degradation of xenobiotic compounds are located on

chromosome and are generally clustered. But in some cases, the genes are located on extrachromosomal genetic elements, plasmids, and transposons.

An understanding of the genetic basis, enzymes, and interaction between the microorganisms and the environment is important for successful implementation of the bioremediation process (Hussain et al. 2009). Furthermore, it will help to develop or genetically engineer strains for better adaptability, high efficiency, and versatility by recruiting heterologous genes to give new characteristics (Pieper and Reineke 2000; Zhang et al. 2005).

9.6 Bioremediation

Bioremediation refers to the process that uses microorganisms (with metabolic capabilities to degrade persistent organic pollutants) or their enzymes to degrade and remove contaminants from the environment. Bioremediation, as a biotechnological tool, has received considerable attention in research due to the fact that it is eco-friendly and involves biotransformation of hydrocarbons and other pollutants by microorganisms. In this process we exploit the ability of microorganisms to degrade/detoxify pollutants from the natural environment and the process is considered better over the physicochemical process as it is efficient, economical, versatile, and environmentally sound. In general the approach is environmental modification such as addition of nutrients, aeration, and even seeding with appropriate degrading organisms (Iwamoto and Nasu 2001; Demnerova et al. 2005). The importance of bioremediation has been increasing in the management of hazardous chemicals such as PCB, TCE, PCE, and BTEX. The principles of bioremediation are based on natural attenuation (bioattenuation), bioaugmentation, and biostimulation (Olaniran et al. 2006). In natural attenuation and biostimulation, we depend upon the natural microflora and enhance their activity by suitable modifications like nutrient availability, aeration, etc. In bioaugmentation the contaminated site is inoculated with microorganisms with desired catabolic capabilities (Fulekar 2005).

9.6.1 Types of Bioremediation

Natural attenuation or bioattenuation is the process of reduction of contaminant naturally as a result of aerobic and anaerobic biodegradation, uptake by plants, physical phenomena such as dispersion, dilution, diffusion, volatilization, sorption, desorption, and chemical reactions (ion exchange, complexation, abiotic transformation). Under suitable conditions natural microflora shall bring about transformation of the chemical pollutants so that it poses no risk or little risk to human health. Natural attenuation occurs in most polluted sites. During the process, the progress of degradation is monitored to ensure that the contaminant decreases. Bioattenuation is used as a cleanup method for underground storage tank sites with

petroleum-contaminated soil and groundwater (Guerin 1999; Okoh 2006). The microbes interact with the contaminant in four different ways:

1. The microorganisms bring about complete degradation of the organic chemicals to CO₂ and water and render them harmless.
2. The chemical is sorbed on the soil particle and thus holds it in place. In this manner, there is no cleanup, but the chemical does not move to pollute the underground water.
3. As the pollutant moves through the top soil, it mixes with groundwater, thereby reducing its concentration.
4. Some contaminants like oil and solvents can undergo evaporation resulting in the reduction of the contaminant in the environment.

Biosorption is an effective biological technique that is just as good as bioaccumulation or biostimulation, and in biosorption either natural resources or microorganisms are used. Biosorption is advantageous over bioaccumulation processes because it is a low-cost process due to the use of industrial and agricultural waste biomass, operates under ambient conditions of pH and temperature as the biosorbent is active, easy to store or use, binding sites have the ability to accommodate a variety of ions, higher degree of rapid toxicant uptake, higher possibility of biosorbent regeneration, and can be reused with possible toxicant recovery. Biosorption is needed for removal of pollutants such as heavy metals and dyes from water. Waste biomass, fungi, bacteria, and algae are known for their biosorption potential. Fungi algae can absorb Cd, Cu, Pb, etc. (Sarkar et al. 2010); grape slicks and other agricultural wastes have been used for biosorption. Thus in situ technologies are promising for a wide range of pollutants and are cost-effective (Das et al. 2007; Sulaymon et al. 2013).

Biostimulation—The natural biodegradation process is slow and can be enhanced by modification of the environment by addition of nutrients such as sources of carbon, nitrogen, and phosphorus, trace elements, and electron acceptors or donors for growth of microorganisms. The addition of substrates such as phenol, toluene, etc. has to be monitored as they are toxic in nature. The addition of lactate resulted in complete conversion of trichloroethane and perchloroethane to ethane. Agricultural practices like cultivation, irrigation composting, etc. enhance the degradation process by increasing aeration and bioavailability of the contaminant in nature (Liliane et al. 2012). Addition of lactate and anthraquinone-2,6 disulfonate (AQDS) enhanced rates of degradation of pentachlorophenol. Comparison of processes of bioattenuation, biostimulation, and bioaugmentation showed that bioaugmentation was most effective and, natural attenuation was more effective than biostimulation of soils contaminated with diesel oil (Bento et al. 2005). On the other hand, results obtained by Liliane et al. (2012) indicated that biodiesel remediation was better in case of biostimulation. This process is expected to become a reliable and safe cleanup technology as natural microflora is used with minimum modification of the natural environment.

Bioaugmentation is the way to enhance biodegradative capacity of the contaminated sites by inoculation of bacteria with desired catalytic capabilities. The basic principle of this intervention is to enhance genetic diversity leading to a wider repertoire of biodegradative reactions.

This is an effective method in which the microorganism cultured individually or as consortium on the particular contaminant in the laboratory is used to seed the site of contamination where bioattenuation and biostimulation is not effective. However, since large population of degradative bacteria is applied at the contaminated site, its effect on the ecosystem and human health must be carefully watched. The injected microbe should either perish or dilute out after the remediation process and should not affect the resident microflora or microecological environment (Gentry et al. 2001). Naturally isolated *Ralstonia europa* KT-1, a phenol utilizing bacterium isolated from the same contaminated site, was used for the first time for bioremediation in 2000 in Japan (Nakamura and Sawada 2000). However, development of microcosm adapted to the particular contaminated site may be a suitable alternative to individual culture. The genetically engineered microorganisms exhibiting enhanced degradative abilities encompassing a wide range of aromatic compounds have potential for bioaugmentation. However, the use of genetically engineered organisms in the environment has its own implications. As such the promising strategy to speed up bioremediation is the use of a combination of bioaugmentation and biostimulation. Both indigenous and exogenous microorganisms could benefit from stimulation by addition of energy source or electron acceptors (Mrozik and Piotrowska-Seget 2010).

9.6.2 In Situ Bioremediation

The in situ and ex situ techniques are used to define whether the treatment of soil and groundwater is undertaken on site, i.e., with minimum disturbance or treatment is applied off site, i.e., the soil is excavated or water is pumped out and bioremediation treatment is undertaken by using suitable microorganisms.

In situ bioremediation is the most desired method of bioremediation as it is carried out in place, no disturbance of the natural environment, and is also cost-effective. In situ bioremediation relies on natural microflora for degradation of contaminants. It may be intrinsic or enhanced and various factors such as soil type, soil characteristics, presence of indigenous microorganisms, type and concentration of contaminant, etc. play an important role. Intrinsic bioremediation does not require any type of amendments or changes in current conditions. Besides biodegradation intrinsic bioremediation may include abiotic processes, viz., dispersion, dilution, sorption, and volatilization (EPA 2000; 2004). Thus the process relies on natural, physical, and biological processes.

Enhanced in situ bioremediation is applied to soil and groundwater by enhancing the growth and metabolic rate of the resident microflora by additives such nutrients, oxygen, or other electron acceptors, or by introducing other suitable amendments. In situ treatment is limited to 30 cm, and in many soils, effective oxygen diffusion

Table 9.14 Comparison of different methods of bioremediation (Vidali 2001)

Type of bioremediation	Benefits	Limitation	Important factor
In situ bioremediation Biosparging Bioventing Bioaugmentation	Cost-effective for soil and water Noninvasive, passive, natural Attenuation	Environmental constraints Long duration Monitoring difficulties	Degradative capabilities of indigenous microorganisms Presence of other pollutants
Ex situ bioremediation Land farming Composting Biopiles	Off-site bioremediation Cost efficient Low cost Suitable for on-site remediation	Mass transfer problem Space requirement Long duration Bioavailability limitation	Environmental parameters, solubility, and distribution of pollutants Geological factors
Bioreactors Slurry reactors Aqueous reactors	Rapid degradation, optimized parameters, enhanced mass transfer, effective use of inoculants and surfactants	Requires soil excavation, High capital cost, high operational cost	Same as above Bioaugmentation Toxicity of amendments, Concentration of contaminants

for effective bioremediation occurs only in a few centimeters of the top layer of soil (Table 9.14).

9.6.2.1 Important In Situ Bioremediation Treatments

Aerobic bioventing is the most common in situ treatment for unsaturated soils by stimulating indigenous bacteria. Low airflow rates provide oxygen in amounts required for biodegradation while minimizing volatilization and release of contaminants to the atmosphere. Air is introduced by air injection wells that push air into the subsurface. Bioventing is suitable for decontamination of simple hydrocarbons and can be used in cases where contamination is deep under the surface. The rates of decontamination depend on the system design, soil gas permeability, soil water content, depth of contamination, and oxygen supply (EPA 2000).

Bioventing technology has been useful for many sites under different conditions, but it has limitation as delivery of oxygen can't be made in soils saturated with water and soils which have low porosity. Soils having oxygen level between 2 and 5 % will not be affected by bioventing because such soils already have enough oxygen to undertake bioremediation. Bioventing is a long-term process and it may take few years to clean up a site. In situ biodegradation involves supplying oxygen and nutrients by circulating aqueous solutions through contaminated soil to stimulate naturally occurring bacteria to degrade organic contaminants. This is useful for soil and groundwater. The technique includes conditions such as the infiltration of nutrient solutions and oxygen as electron acceptor for treatment of groundwater (De Wilde et al. 2007).

Air Sparging is the process of injection of air under pressure below the water table to increase groundwater oxygen concentrations and enhance rate of biodegradation of contaminant by natural microflora. The pumped air mixes with aquifer, the aerated water migrates through the zone of contamination, and the indigenous bacteria degrade the contaminant (EPA 2000).

Oxygen injected below the water table volatilizes contaminants that are dissolved in groundwater, existing as a separate aqueous phase. The volatilized organic compounds migrate upward in the vadose zone where they are removed by using soil vapor extraction techniques. Air sparging also promotes degradation by increasing oxygen concentration in the subsurface, stimulating aerobic biodegradation in saturated and unsaturated zones. This is suitable at sites where groundwater and saturated soils are contaminated with volatile organic compounds (VOCs), semi-volatile (SVOCs), and/or nonvolatile aerobically biodegradable organic contaminants such as gasoline, diesel, jet fuel, oils, grease, and highly chlorinated compounds.

Biosparging is similar to air sparging, but this involves injection of gas phase nutrients under pressure into the saturated zone to promote biodegradation. This enhances aerobic bioremediation and is suitable for contaminants such as gasoline components (benzene, toluene, ethyl benzene, and xylene), VOC, and SVOCs. By using biosparging up to 75 % removal of contaminant was achieved.

Bioaugmentation is the remediation process involving indigenous or allochthonous wild-type or genetically modified microorganisms for treatment of sites contaminated with hazardous organic chemicals. This process suffers an account of the following: (1) nonindigenous cultures rarely compete well with the indigenous population, and (2) soils with long-term exposure to biodegradable organic compounds result in development of degradable abilities in indigenous populations.

9.6.3 Ex Situ Bioremediation

Ex situ bioremediation technologies are used for bioremediation of contaminants for various solids, solid–liquid mixtures, and liquids. The process may involve excavation of soil and treat it at a different site. The treatment methodologies involved are as follows.

9.6.3.1 Land Farming and Composting

This is a simple technique in which the contaminated soil is excavated and spread over a prepared bed and degradation of the contaminant is carried out under increased aeration by periodical tilling or mechanical mixing. Normally indigenous microorganisms are used. The land treatment generally entails 8–12-inch layer of soil.

Composting is the technique that involves combining contaminated soil with nonhazardous organic amendments such as manure or agricultural waste. This supports the development of rich microbial population characterized at higher

temperature for growth. Composting takes place between 40 and 50 °C and the thermophilic microorganisms are active at this temperature. Proper conditions of oxygen, moisture, and temperature are maintained. Frequent mixing is carried out for aeration and surface irrigation is done for maintaining moisture level. Temperature is maintained by mixing, irrigation, and air flow. Microorganisms degrade organic matter to CO₂, water, and humic acid as end products. Degradation is brought about by a consortium of aerobic organisms, predominantly bacteria, fungi, and actinomycetes. They break down complex organics to smaller molecules which are used as carbon and energy sources. Compost is either dark brown or black and is not soluble in water. Composting has been successfully applied to soils and bio-solids contaminated with petroleum hydrocarbons, e.g., fuels, oils, grease, solvents, chlorophenols, pesticides, herbicides, and nitro-aromatic explosives.

9.6.3.2 Biopiles

Biopiles are a hybrid of land farming and composting. Specially engineered cells are constructed as aerated compost piles. These are used for surface contamination with petroleum hydrocarbons. These are the improved versions of land farming in which physical losses by leaching and volatilization are controlled. Biopiles provide suitable environment for indigenous microorganisms (Castillo et al. 2008).

9.6.3.3 Bioreactors

Slurry reactors or aqueous reactors are used for ex situ treatment of soil and pumped water from contaminated wells/bore wells. Slurry bioreactor is a containment vessel and apparatus used to create three-phase suspension, aeration, and mixing environment to increase the bioremediation rate of soil-bound and water-soluble pollutants, usually by indigenous microorganisms. The rate and extent of degradation in the bioreactor is higher than the in situ or solid-phase system. Bioreactors have proved most effective for soil, sediments, and solid remediation. Soils contaminated with petroleum showed 80 % conversion in 10 days in a slurry reactor which required 150 days under fixed bed conditions. Enhanced contaminant was due to increased bioavailability of the contaminant and aeration. Manipulation of the reactor parameters can result in faster degradation.

9.6.4 Bioaugmentation

Bioaugmentation is an important aspect of bioremediation which improves the biodegradative capacities of contaminated sites by introduction of single strains or consortia of strains with desired catalytic capabilities or genetically engineered microorganisms encompassing a wide range of aromatic-hydrocarbon degradative genes from other microorganisms (Hamdi et al. 2007). However, it has been suggested that bioaugmentation approach should be applied when bioattenuation and biostimulation have not been successful and the soils (1) have very low or non-detectable number of contaminant-degrading bacteria, (2) the contaminant requires multiprocess bioremediation and is toxic to microbes, and (3) the site to

be treated is small and cost of treatment by physicochemical methods is higher than bioaugmentation. Furthermore, bioaugmentation is recommended for areas contaminated with compounds requiring long acclimation or adaptation period of time (Forsyth et al. 1995; Vogel 1996; El Fantroussi and Agathos 2005).

9.6.4.1 Microorganisms for Bioaugmentation

Selection of suitable strains of a consortium of different microorganisms is important for successful bioremediation process. Important features are fast growth, easy to be cultured, and ability to tolerate high concentration of pollutant and to withstand wide range of environmental conditions. Microorganisms that have been maintained and specifically adapted or modified for bioaugmentation should be preferred (Singer et al. 2005; Thompson et al. 2005). For example, for remediation of sites contaminated with PAHs and biphenyls, it is necessary to use strains producing surfactants to make the pollutant more accessible (Gentry et al. 2004; van der Gast et al. 2003). Different approaches for selection of microorganisms for bioaugmentation are:

- (a) Isolation of indigenous bacteria from the contaminated site, and after culturing in the laboratory, it may be preadapted to same contaminated site.
- (b) Selection of appropriate microorganism from the sites contaminated with same/similar contaminant that is present in the soil to be treated.
- (c) The consortia of microorganisms from the contaminated soils may be developed for bioaugmentation. Consortia of aromatic-degrading bacteria have been found to be more useful than single strain (Ghazali et al. 2004). Most of the naturally evolved bacterial strains and isolated from aromatic-hydrocarbon contaminated areas or industrial wastewater treatment plants have been found to be suitable for bioaugmentation (Dong et al. 2008; Cordova-Rosa et al. 2009; Adekunle 2011).

Members of genus *Pseudomonas* have been extensively used for bioaugmentation studies and other groups of bacteria used for the purpose are *Flavobacterium*, *Alcaligenes*, *Sphingomonas*, *Burkholderia*, *Achromobacter*, and *Rhodococcus* (Dan and Edward 2001; Mrozik and Piotrowska-Seget 2010). Most of these bacteria are versatile and degrade a wide spectrum of compounds.

9.6.4.2 Bioaugmentation with *Pseudomonas* as a Single Strain

Pseudomonas and related bacteria have been used as pure cultures for biodegradation of aromatic compounds, e.g., *P. putida* ZWL73 has been used for decontamination of 4-chloronitrobenzene (4CNB) in soil microcosms (Niu et al. 2009). *Sphingobium chlorophenolicum* degraded up to 80 % of pentachlorophenol (PCP) of the 100 mg kg⁻¹ soil dry weight in 2 weeks of incubation compared to 5 % in the uninoculated samples (Dams et al. 2007). Similarly, fenitrothion degradation was studied by using *Burkholderia* species. There was complete utilization in 2 weeks while uninoculated samples showed 30 % degradation (Hong et al. 2002). Psychrotolerant *Acinetobacter johnsonii* B-2-2 was able to degrade 75 % of

hydrocarbon compared to 35 % in case of natural microflora. Combination of *Pseudomonas* H1 and *Ralstonia eutropha* IMP134 showed enhanced degradation of 2,4-D (Roane et al. 2001).

9.6.4.3 Consortia of Microorganisms for Bioaugmentation

Attempts have been made to develop consortia of bacteria or combination of bacteria and fungi for enhanced degradation of hydrocarbons. A combination of biphenyl-degrading *Pseudomonas* sp. strain JHK, a chlorinated benzoate degrading *P. putida* PaW1, and a chlorocatechol degrading *Pseudomonas* sp. B13 was used to degrade Aroclor 1221 (a mixture of PCBs) in a soil microcosm. Complete degradation of Aroclor 1221 was observed in inoculated sterile soils due to their different metabolic activities; however in non-sterile soil, formation of 4-chlorobenzoic acid adversely affected the degradation of Aroclor 1221 (Havel and Reineke 1992). Yu et al. (2005) studied biodegradation of a mixture of fluorene, phenanthrene, and pyrene by a bacterial consortium comprising of *Rhodococcus* sp., *Acinetobacter* sp., and *Pseudomonas* sp. They observed that addition of consortium significantly enhanced removal of phenanthrene (97 %) and fluorene (99 %) in 2 weeks of incubation while only 10 % pyrene was degraded under natural conditions. However, all the PAHs were degraded in 4 weeks.

A consortium of *P. mendocina* PC1 and three strains of *P. fluorescens* PC18, PC20, and PC24 was used for degradation of phenolic compounds in leachate and soil-contaminated microcosm (Heinaru et al. 2005). Bacterial population analysis showed that *P. fluorescens* PC20 and PC18 dominated in oil-amended soil, whereas *P. mendocina* PC1 and *P. fluorescens* PC24 dominated phenolic-leachate microcosm. The leachate microcosm contained high proportion of bacteria possessing meta- and ortho- pathways for phenol and *p*-cresol degradation. Domination of strains PC20 and PC18 in the oil-amended soil was due to their ability to degrade naphthalene and salicylate. Similar observations have been reported for other consortia (Jacques et al. 2008; Ghazali et al. 2004; Bara Caracciolo et al. 2013).

9.6.4.4 Immobilization and Delivery of Cells for Bioaugmentation

For bioaugmentation to be effective, it is necessary that proper number of cells is introduced and maintained. Thus the microorganism must be delivered in a manner that it is uniformly distributed and their shelf life and activity are maintained. Thus an ideal carrier for microorganisms must provide a protective niche and temporary nutrition to the microbe and be nontoxic both to the inoculant and resident microflora. Liang et al. (2009) reported that the use of activated carbon enhanced hydrocarbon degradation which could be attributed to improved diffusion of oxygen, nutrient mass transfer, and water holding capacity of the bio-carriers.

Immobilization of cells offers protection of microorganisms from suboptimum environmental conditions like pH, temperature, presence of toxic substances, as well as reduces competition with indigenous microflora. Immobilization also enhances stability of cells and plasmids (Cassidy et al. 1996). Various types of materials such as dextran, agar, agarose, alginate, chitosan, polyacrylamide, and

k-carrageenan can be used as immobilization medium (Cassidy et al. 1996; Gardin and Pauss 2001).

Cunningham et al. (2004) used polyvinyl alcohol cryogelation for entrapment of microorganisms and used for cleanup of soil contaminated with diesel. Comparison of the bioaugmentation by free cells, biostimulation, and immobilized bioaugmentation revealed that immobilized system was most effective for diesel degradation. Degradation of 2,4-D was studied in immobilized cell bioreactor and compared with free living cells. Cells of *P. putida* P₁ in Ca-alginate were more effective, and faster rate of degradation of 2,4-D was observed under controlled conditions (Kocher and Kahlon 2003). Wasi et al. (2011) reported that *P. fluorescens* SM cells immobilized with Ca-alginate were effective to remove toxicants such as phenols, pesticides, and heavy metals. A comparison of the free living and immobilized cells of *Pseudomonas putida* P₁ showed enhanced degradation by cells immobilized in Ca-alginate beads under laboratory conditions (Singh and Kahlon 1992).

9.6.5 Rhizoremediation

Plant-microbe interactions comprising of rhizosphere (soil), rhizoplane (root surface), and root tissue with some endophytes offer a useful system for bioremediation of soil contaminated with persistent organic pollutants (Chaudhry et al. 2005; Kuiper et al. 2004). The plants help in the spread of bacteria through the soil by their root system, even to the deeper layers. The plant exudates are a rich source of water-soluble nutrients including carbohydrates, amino acids, organic acids, alcohols, flavonones, phenolics, etc. which promote growth of bacteria and thus create a niche for microbial growth and higher metabolic activity in the rhizosphere. The bacteria with degradation capacity for pollutants help degrade the pollutants and thus protect the plant from the toxic effects of herbicides, insecticides, and other pollutants.

Root systems of some wild-type grasses, legumes like alfalfa and cereal crops wheat, barley, and some tree plants have been found to have useful interactions with soil microorganisms for degradation of variety of pollutants such as 2,4-D, phenols PAH, and chlorinated aliphatic and aromatic compounds. The root systems of these plants are highly branched and harbor large populations of microorganisms. The bacteria that are capable of degrading polluting chemicals will flourish and thus dominate the microbial populations in the rhizosphere (Liu and Suflita 1993; Dubey and Fulekar 2013). Studies have revealed that *Pseudomonas putida* and *P. fluorescens* which are well-known root surface colonizers are the predominant bacteria. *Pseudomonas fluorescens* also act as biocontrol agents and protect the plant against soil borne plant pathogens. Metabolically *P. putida* KT2440 is very versatile and an efficient root colonizer (Narasimhan et al. 2003).

It was observed that the consortia of pollutant-degrading bacteria in the rhizosphere were necessary for efficient degradation. Maybe they supplement different pathways or each may carry out different parts of catabolic degradation route.

Isolation of *Pseudomonas putida* PCL1444 from grass rhizosphere also resulted in isolation of *P. putida* PCL1445 as a coculture. *Pseudomonas putida* PCL 1445 failed to grow on mediums containing naphthalene as sole source; however, when this was co-inoculated by metabolic mutants of *P. putida* PCL 1444 blocked at different steps, it showed growth on naphthalene when a mutant of *P. putida* PCL 1444 possessing *doxG* (*nahC* homolog, involved in the conversion of 1,2-dihydroxy-naphthalene into *cis-o*-hydroxybenzal-pyruvic acid) gene was functional (Okuta et al. 2004). Therefore, the selection of suitable bacteria or communities that are able to sustain and proliferate on the root system of the plant will be useful for bioremediation in the natural system.

9.6.6 Factors Affecting Bioremediation

Bioremediation is a complex process involving many factors, viz., (1) the existence of native microbial population capable of degrading the pollutants, (2) the nature and type of contaminant and its availability to microbial population, and (3) the environmental factors, viz., the soil type, temperature, pH, presence of oxygen, and other electron acceptors and nutrients. All these make the process optimization and control difficult.

1. *Microbial populations capable of degrading pollutants*—Microorganisms are characterized with an incredible metabolic and physiological versatility and as such occupy hostile ecological niches and exploit the variety of xenobiotics as sources of carbon and energy. Members of the genus *Pseudomonas* are particularly important and can metabolize an array of noxious organic chemicals. It is therefore desirable to exploit this natural capacity of the microorganisms to develop effective biotechnological processes to eliminate toxic chemical pollutants. Adaptability of microbes and other biological systems makes these suitable to degrade and remediate environmental hazards. Broadly these processes may be classified as aerobic and anaerobic depending upon the requirement of oxygen.

In *aerobic mechanism*, organisms using oxygen as terminal electron acceptor, e.g., *Pseudomonas*, *Sphingomonas*, *Alcaligenes*, *Rhodococcus*, *Mycobacterium*, etc. are used. These generally degrade pesticides and hydrocarbons including alkanes, aromatics, and polycyclic compounds.

In *anaerobic mechanism*, the process proceeds in the absence of oxygen. The anaerobic bacteria bring about dehalogenation of PCBs, TEC, and chloroform, etc. in river sediments and sludge. Bio-methanation is also used for anaerobic treatment of agro-industrial wastewaters.

Besides these methylotrophs is the group of bacteria that utilizes methane as source of carbon and energy. The key enzyme, methane monooxygenase, has a broad substrate range and is active against a wide range of compounds such as trichloroethylene and 1,2 dichloroethane. Ligninolytic fungi, such as

Phanerochaete chrysosporium, have the ability to degrade a diverse range of toxic environmental pollutants such as dyes, straw, and sawdust, etc.

The factors affecting microbial degradation of organic compounds may include inhibition of enzyme activity and growth of degrading organisms. This inhibition may result from competition between microorganisms for limited carbon sources, antagonism among different microorganisms, and predation by protozoa and phages. The rate of degradation depends upon the concentration of contaminant and the amount of catalyst, in other words the population size. Furthermore, the affinity of the enzyme for the substrate is also important. Other factors affecting the enzyme activity are temperature, pH, and moisture. For degradation, the contact between the bacterial cell and the contaminant is important and in nature the bacteria and the chemical may not be uniformly spread and may be adsorbed on the surface of the particle. Some bacteria like *P. aeruginosa* produce surfactants and this helps to reduce the surface tension on the cell, thus facilitating contact with the contaminant.

2. *Environmental factors*—Soil type and organic matter content play an important role as the contaminant may be adsorbed and absorbed on to the soil particle rendering it unavailable to microorganisms for degradation. Similarly the variations in porosity of saturated and unsaturated zones influence the movement of fluid contaminant, bacteria, and exchange of gases such as oxygen, carbon dioxide, and methane affecting overall rate of degradation. Besides the availability of the appropriate nutrients is important because the microorganisms may be present in the contaminated site, but their number may not be sufficient for effective degradation of contaminant. Thus their growth and activity must be stimulated by providing extraneous nutrients (Ortiz-Hernández et al. 2011).

Physical features such as temperature, pH, and moisture also affect growth and activity of microorganisms. Generally, the optimum range of these conditions is very narrow. Requirement of oxygen will depend upon whether the process is aerobic or anaerobic. Hydrocarbons are readily degraded under aerobic conditions and chlorinated hydrocarbons compounds are degraded more efficiently under anaerobic conditions.

9.7 Biotechnology and Emerging Technologies

Pseudomonas sp. and many other bacteria have evolved plasmid-borne genes for peripheral pathways and degradation of xenobiotics. Plasmids also provide a means for fast spread of genetic information among populations by horizontal gene transfer, and plasmids usually carry insertion sequences rendering sufficient functional flexibility. Catabolic pathway encoded on plasmids includes TOL/pWWO, TOL/pWW53, TOL/pDK1, PBH/pWW110, NAH/NAH7, PHE/pV1150, transposons, or other mobile/integrative elements. Genetic manipulation of the plasmid-borne genes provide a great opportunity to enhance degradative potential of microorganisms (Sayler and Ripp 2000; Kocher and Kahlon 2003; Gentry

et al. 2004; Mrozik et al. 2005; Mrozik and Piotrowska-Seget 2010; Wasilkowski et al. 2012).

With the development of recombinant DNA techniques, a large number of genetically modified microorganisms for degradation of hydrocarbons have been studied and compared with the parent/indigenous microflora for bioaugmentation of the remediation process (Lovely 2003; Menn et al. 2010).

Pseudomonas fluorescens HK44 has been allowed to be evaluated for applicability in naphthalene contaminated soil. *P. fluorescens* strains HK 44 contains PUK21 plasmid carrying a transposon Tn4431 insertion into NAH7 plasmid from *P. fluorescens* 5R. The transposon originated from *Vibrio fischeri* and carried *lux CDABE* gene cassette for bioluminescence as a marker. Genes for naphthalene degradation and *lux* gene cassette are under the common promoter, thereby resulting in simultaneous degradation and luminescent signal (Saylor and Ripp 2000).

Another strain *P. putida* KT2442 (pNF142::TnMod-OTc) able to degrade naphthalene was constructed from three strains: *E. coli* S17-1 with pTnMod-OTc plasmid carrying tetracycline resistance, *Pseudomonas* sp. 142 NF (pNF142) able to degrade naphthalene, and *P. putida* KT2442 with *gfp* (green fluorescent protein) gene located in the chromosome. The recombinant bacteria could degrade naphthalene and transfer PNF::TnMod-OTc plasmid to autochthonous bacteria (Filonov et al. 2005; Perez-Pantoza et al. 2008).

4-chlorobenzoic acid is the major stable intermediate in the degradation of PCB and DDT. Strain *P. putida* Paw340 (pDH5) was constructed by cloning *pcb* genes into artificial plasmid pDH5 which was then introduced into *P. putida* Paw340 cells (Massa et al. 2009). A number of strains of *P. fluorescens* with the plant growth promotion properties have been genetically engineered so that they degrade the toxic chemicals in the rhizosphere as well as promote plant growth by colonizing the plant roots (Heuer et al. 1995; Yang et al. 2011). *Pseudomonas fluorescens* strains for degradation of biphenyl, PCBs, 2,4-dinitrotoluene, and phenol have been constructed in addition to plant growth promotion. A number of the genetically modified strains with catabolic properties for bioaugmentation are listed in Table 9.11.

Endophytic strain of yellow lupine *B. cepacia* LS.2.4, *B. cepacia* BU0072, and *P. putida* VM1441 (pNAH7) were genetically modified to protect the plants from toxic effect of toluene, toluene, and naphthalene, respectively (Barac et al. 2004; Taghavi et al. 2005; Germaine et al. 2009). The approach of using the plant-associated endophytic as well as rhizospheric PGPR seems a very plausible solution to remediation of contaminated soils.

The technique of immobilization of cells and nanoparticles can prove useful for uniform distribution of microbial inoculants for bioaugmentation and enhancement of the process of bioremediation. Immobilized cells and enzymes may also be used in the specific bioreactors for degradation of pollutants (Kocher and Kahlon 1999; Cassidy et al. 1996; Cunningham et al. 2004).

Bioremediation technologies which are cost-effective can be further improved by integrating with physicochemical technologies (Meghraj et al. 2011; Shah

2014). In this context the use of emerging technologies of fuel cells, nanoparticles, transgenic plants, and microbes and photoheteromicrobial consortia approaches has entered a new phase in environmental remedial systems and is expected to give birth to new era of green biotechnology with the use of novel methods for in situ bioremediation (Eapen et al. 2007). This will bring about rapid + reliable + low-cost + low-risk-based contaminant cleanup technology.

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Pseudomonas-Plant Interactions I: Plant Growth Promotion and Defense-Mediated Mechanisms

10

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Contents

10.1	Introduction	420
10.2	Beneficial Interactions of Plant Growth-Promoting Rhizospheric Microorganisms . . .	422
10.2.1	<i>Pseudomonas</i> Established Rhizospheric Competency	423
10.2.2	Chemotactic Responses During Competitive Colonization	426
10.2.3	Physiological-Induced Attachment to Plant Roots	427
10.2.4	Chemical-Induced Mechanisms of Synthesis	429
10.3	Biologically Induced Mechanisms of Resistance	432
10.3.1	Phytopathogen Suppression by Siderophores	436
10.3.2	Phytopathogen Suppression by Antibiotics	438
10.3.3	Phytopathogen Suppression by Phenazines	440
10.3.4	Phytopathogen Suppression Through Induced Systemic Resistance	441
10.3.5	Bioinsecticidal Activity of <i>Pseudomonas</i>	444
10.4	Plant Growth Regulators: Phytohormones	447
10.5	Engineering Transgenic Plants with Replicase- and RNA-Mediated Resistance . . .	448
10.5.1	Current Development in Transgenic Crops	450
10.6	Conclusions	451
	References	451

Abstract

Bioaugmentation, biostimulation, and biocontrol using *Pseudomonas* spp. have shown to be very effective in promoting growth, resistance, and competency through chemical, biological, and physical stimulation of various growth factors, systemic signaling moieties, and intercommunal interactions. Nutrients or exudates and electrons acquired through feedback mechanisms between the

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host plant and *Pseudomonas* spp., mutual or synergistic in nature, promote nitrogen fixation and phosphate solubilization, releasing secondary metabolites, such as flavonoids, phytohormones, and ACC and IAA compounds, reflecting their role as plant growth regulators. Other important characteristics of *Pseudomonas* spp. include ecological inhibition of phytopathogens through the production and secretion of antimetabolites, site-specific recombinase enzymes, siderophores, antibiotics, and a signaling cascade for induced systemic resistance. Various *Pseudomonas* spp. have been identified for their role in biological suppression and growth promotion, which include, but are not limited to, fluorescent pseudomonads such as *P. fluorescens*, *P. putida*, *P. aeruginosa*, *P. vancouverensis*, *P. jessenii*, and *P. stutzeri*, among others. While several *Pseudomonas* spp.-based commercial products are in the market as biofertilizers and bioinoculants, engineering transgenic crops has also shown promises, promoting biotic resistance to pests, increased herbicide tolerance, enhanced disease resistance, and affective control against abiotic and environmental stresses.

10.1 Introduction

Mass production and intensive agriculture are complexed in a highly competitive and revolving industry. Pulse crop farming and orchard, rice field, and cotton farming are the essence of economic stability for many developing countries (Ramarethinam et al. 2005). The agriculture push coupled with increased populations and consumer demands has left many farmers seeking new methods of providing sustainable products. Aggressive production, due to increasing demands for cereals and tuber crops such as potato, cassava, sweet potato, sugar beet, sugarcane, and others, result in intensive farming, coupled by poor soil infrastructure which have led to postharvest losses accounting to 25–30 % due to microbial-induced spoilage (Pandey et al. 2011). The 1970s green revolution brought forth the concerns of soil infertility as a result of years of invasive chemical pollution and insecticides heavily taxing the soil and its ecology. In addition, traditional tilling and overgrazing practices hinder nutrient recycling through the erratic expenditure of resources to accommodate for high demand single harvests (Khan and Parmar 2013).

Soil infertility and degradation in India alone have resulted in an 18.5 % record of uncultivable top soil where of the 329 million ha of geographical land, 175 million ha is considered to be partially degraded due to such aggressive means of agriculture (Sharma 2005; Bhadauria et al. 2010). To cope with the ever-increasing demand, biotechnology has fused traditional organic methods of harvest with key elements that promote rhizobial competency in aims to create more biological sustainable, self-regulating, competent, and interactive rhizobacterium. These methods of rhizobial modification for plant growth promotion are known as bioaugmentation, biostimulation, and biocontrol. The supplemental strains interact as competitive or synergistic associates among the native consortium to promote

active metabolism and colonization while reducing or eliminating root rot or infection caused by pathogen and parasitic microorganisms (Nautiyal 2000). It is believed that one billion bacterial species actually exist on Earth, of which, only 5000 species from the environment have been identified (Hunter-Cevera 1998; Curtis and Sloan 2004). Furthermore, microorganisms on Earth and in soil may harbor up to 10 billion microorganisms per gram and that 1 g of soil may contain up to 4000 different bacterial genomic units, accounting for the mass diversity and competition within the rhizosphere (Forney et al. 2004; Blagodatskaya and Kuzyakov 2008). In retrospect, microorganisms in the soil constitute for less than 0.5 % (w/w) of the soil mass. However, these microfloras ultimately determine the interactions in the soil, forming a dynamic forum of beneficial microorganisms interacting among themselves, others, and the plants they seek to protect and promote growth (Dudeja et al. 2011).

Plant growth-promoting rhizobacteria (PGPR) and endophytes are naturally occurring biocontrol agents. PGPR improve root and shoot growth, nodulation, colonization, chemical acquisition, sporulation, and surface-activated defense mechanisms of plants (Wang et al. 2005; Rokhzadi et al. 2008). Endophytes could be bacterial or fungal forms that exist entirely asymptotically within the living host plant tissues in the interspaces between the epidermal cells, below collapsed or within epidermal cells, and/or inside the intercellular spaces in the root cortex (Kalia and Mudhar 2011). The endophytes interact with external PGPR that have entered the plant through cracks or site of injury to the plant root interior, by an interplay of chemical signals between the endophyte and the root nodulating PGPR (Gray and Smith 2005). This activity enables the inhabitation of host tissues to enhance systematic resistance or block the receptor sites for attachment or adsorption of the pathogen (Harrier 2001). Species of bacteria that have been identified for their numerous beneficial attributes in biocontrol, biostimulation, and bioaugmentation are those belonging to the genus *Pseudomonas* spp. The *Pseudomonas* spp. is a motile bacterium, with one or several polar flagella, non-sporulating rods with gram-negative reaction and 58–69 % GC content. They are catalase positive and chemoorganotrophic, which allows for metabolic respiration to ensure using oxygen and sometimes nitrate as the terminal electron acceptor (Palleroni 2008). Within the *Pseudomonas* spp., a large number of bacteria belonging to the rRNA group I of the fluorescent pseudomonads are capable of producing fluorescent pyoverdine siderophore(s) including *P. aeruginosa*, *P. syringae*, *P. putida*, and *P. fluorescens* (Bossis et al. 2000). Fluorescent pseudomonads have a profound ability to circumnavigate through the rhizosphere, acquire necessary nutrients and electrons or exudates, and display a feedback mechanism where the interactions among the host plant and native bacterium, mutual or synergistic in nature, assist in each other's survival. In the rhizosphere, *Pseudomonas* spp. are often found near the root surface (rhizoplane) forming microcolonies in the rhizosphere or discontinued biofilms in the grooves between epidermal cells, forming endophytic colonies (Duijff et al. 1997). This form of biological activity is rather aggressive in terms of colonization through elimination of readily available exudates for non-desirable organisms. Through their association with the plant,

stimulation and colonization in the soil are beneficial; however, *Pseudomonas* spp. can actively protect against plant pathogens using chemical signals and agents to assist in protecting plants from rhizosphere-dwelling phytopathogens (Coullero et al. 2009). Such factors include flavonoids, phytohormones, enzymes, siderophores, antibiotics, and induced systemic resistance through signal-mediated induction (Saharan and Nehra 2011). These diverse mechanisms of protection, assimilation, and growth promotion by *Pseudomonas* spp. are being exploited in numerous projects all around the world with the aim to launch more sustainable, robust, self-controlling, and noninvasive inoculums. In this chapter, we will examine and understand the role *Pseudomonas* spp. play in biocontrol, growth promotion, and rhizobial competency and discuss the mechanisms which make *Pseudomonas* spp. such a desirable PGPR.

10.2 Beneficial Interactions of Plant Growth-Promoting Rhizospheric Microorganisms

Interactions among the biological community are described as complex, multifaceted, and always evolving. Biological diversity or biodiversity encompasses such organisms/animals and leguminous species, their genetic material, and their corresponding ecosystem, with the diverse interactions occurring in the ecosystem between species and genetically diverse levels (Fontaine et al. 2003; Lynch et al. 2004). Microbial communities interact among one another for survival, proliferation, and colonization, through competitive, synergistic, neutralistic, associated, or antagonistic interactions (Lynch et al. 2004).

Through chemical breakdown and uptake of essential nutrients, a dominant species, such as PGPR bacteria, will encourage growth and proliferation of plant parameters as well as reduce invasion from competitors by inducing mechanisms that readily fix nitrogen, synthesize siderophores for iron utilization, and promote the synthesis of phytohormones (Glick 1995; Saharan and Nehra 2011). Either of these mechanisms used by PGPR can provide conditions that stimulate secretion of root exudates from host plants, thereby encouraging colonial growth and proliferation of the PGPR subsequently, mandating resistance, and aiding from root rot and soilborne pathogens. PGPR such as *Pseudomonas* spp. have been described by many as the most effective root-colonizing group of soil microorganisms (Lugtenberg et al. 2001a, b; Haas and Defago 2005). They are nutritionally diverse, have a high growth rate in soil, interact competitively in the rhizosphere, and provide feedback to the host through increased productivity and uptake while reducing/weakening and even outright deleting the competing organism. Inhibition and activity can occur through direct means of production of plant growth-promoting factors, such as the production of auxins, cytokines, gibberellins (Noori and Saud 2012), indoleacetic acid (IAA), phosphate solubilization, ACC deaminase activity, root elongation, degradation of toxic compounds, and biological control against phytopathogens (Wahyudi et al. 2011; Bai et al. 2003; Bano and Musarrat 2003; Vasanthakumar and McManus 2004). Indirect methods of

inhibition and plant growth are shown through the production of secondary metabolites, such as siderophores, antibiotics, and HCN that stunt the growth and incidence of parasitic contact (Wahyudi et al. 2011).

10.2.1 *Pseudomonas* Established Rhizospheric Competency

Pseudomonas spp. have shown resilience in the growing (no pun intended) agricultural field, providing justifiable means of sustainability, stimulation, and remediation. *Pseudomonas* spp.'s interaction and uptake are essentially the ultimate determining factors for successive colonization and continuous proliferation within and among the indigenous rhizobacteria. Physiologically, the most effective *Pseudomonas* spp. strains are those which are gram negative and motile, promote root and shoot elongation, and are genetically affixed with various phytobeneficial traits (Noori and Saud 2012). The concentration of bacteria surrounding the rhizosphere as per gram of soil compared to that of the bacteria found existing in aggregates dispersed throughout the soil is generally found at much higher folds (Lynch 1990). This accounts for the high levels of metabolic activity occurring within root regions. Nutrients such as atmospheric nitrogen, phosphorus, and carbon are readily available in agro-rich regions. Artificially introduced rhizobacteria initially establish high population densities, forming continuous feedback between plant and endophyte/rhizobacteria, and however tend to decline after the initial inoculum due to high levels of microflora and sudden depletion of resources (Haas and Defago 2005). Furthermore, plants can actively select the root-colonizing microorganism through the induction of resistance mechanisms and through what is known as rhizodeposition, selectively secreting specific exudates components required for the specific microbial benefactor (Singh et al. 2004; Oksinska et al. 2011). This mechanism of interaction and supply establishes a feedback-mediated mechanism of rhizospheric competency.

Pseudomonas spp. in particular resonate traits involved in the ecological competency, such as quorum sensing, biofilm formation, production of antifungal metabolites, attachment to roots, chemotaxis, site-specific recombinase activity, and uptake and catabolism of root exudates (Lugtenberg et al. 2001a, b; Mavrodi et al. 2001). Several strains of fluorescent *Pseudomonas* species have been shown to improve health and growth and to play an important role in the natural suppression of *Fusarium* wilts, against take-all disease of wheat and against tobacco root rot (Ghirardi et al. 2012).

To understand competency, the relationship between the level of disease suppression and the population density of *Pseudomonas* strains in the rhizosphere of the corresponding plant must first be established (Lugtenberg et al. 2001a, b; Bull et al. 1991). One of the most commonly used strategies to identify traits involved in rhizospheric competency involves the generation and characterization of mutants of *Pseudomonas* strains defective in a specific phenotypic trait (Lugtenberg et al. 2001a, b). Another is a population-based approach to uncover the traits involved in the rhizosphere in the presence of indigenous microflora associated

with roots and bulk soils. This method discriminates traits between the two populations (Clays-Josserand et al. 1995; Latour et al. 2003; Lemanceau et al. 1995). Microarray and in vivo expression are other strategies used to collectively reveal numerous competency traits including flagella, O-antigenic side chain of lipopolysaccharides, nitrate reductase, phenazines, siderophores, surfactants, amino acids, transport metabolism, and oxidative stress resistance (Ghirardi et al. 2012). *Pseudomonas fluorescens* implication in the rhizosphere adaptation has been evaluated using a model strain approach that consisted of comparing rhizosphere competitiveness of model strain *P. fluorescens* C7R12 to that of mutants affected in their ability to synthesize pyoverdine and/or nitrate reductase (Mirleau et al. 2000, 2001). Furthermore, a collection of 23 *Pseudomonas* strains and species was evaluated for their ability to colonize the rhizosphere of tomato plants grown in non-sterile soil in the presence of the indigenous microflora. Competency characteristics in the rhizosphere would theoretically reflect a number of phenotypic traits for survival including those capable of substrate utilization, nitrogen dissimilation, siderophore-mediated iron acquisition, and the production of antibiotics and *N*-acylhomoserine lactones (Ghirardi et al. 2012). Strains were evaluated as rifampin-resistant derivatives on tomato seedlings with low iron bioavailability (*Lycopersicon esculentum* Mill. cv.H63-5) cultivated in soil of Châteaurenard in France.

Five tomato seedlings were transferred to a microcosm subject to the testing conditions, inoculated with mutant bacteria at a density of 10^7 CFU/g dry soil grown over a period of 22 days in a growth chamber under 16 h light + 25 °C and 8 dark + 22 °C. Subject to the time trial, bacteria were extracted, diluted, and grown on KB agar medium supplemented with rifampin (100 mg/l) and cycloheximide (100 mg/l) and expressed as CFU/g dry soil. After survival rate analysis of group 1 and 2, 12 of the 23 strains showed limited survival rates, while the others, groups 3, 4, and 5, showed moderate to high survival rates, with the highest reflecting group 5 strain *Pseudomonas fluorescens* CTR212. This delineation reflects the ability of each group to actively compete in the environment, corresponding to the phenotypic clusters 2, 4, and 8, dissimilating abilities (TDe of available nitrogen), siderovars, and by the origins of MIC of EDDHA at 125–250 ppm. Overall, *Pseudomonas lini* and *Pseudomonas fluorescens* showed adequate survival rates, while *P. jessenii* and *P. putida* were less desirable, as the best colonizers reflected an ability to be less susceptible to iron starvation and produce specific siderovars, such as pyoverdine, to instigate siderophore-mediated iron uptake in the limited iron Châteaurenard soil and, consequently, natural suppression of *Fusarium* wilt. This behavior was also shown to be involved in the rhizosphere competence of *Pseudomonas fluorescens* strain C7R12 (Mirleau et al. 2000). As the TDe strains were less likely to chelate iron, the competitive ability was subject to reduce nitrogen oxides (as final electron acceptors) in less oxygen available rhizospheres thus contributing to more efficient colonization and competence in the rhizosphere (Mirleau et al. 2001). Furthermore, the ability to produce the phenazines gave the competitive *Pseudomonas* strains an advantage under iron-limiting conditions to be

redox-active antibiotics and assist in iron mobilization (Dietrich et al. 2008; Hernandez et al. 2004; Price-Whelan et al. 2006; Wang and Newman 2008).

Similar approaches studying competency using population dynamics were conducted with wild-type *Pseudomonas* sp. NBRI9926 strain and *Rhizobium* strains are equipped with biological control against *Fusarium oxysporum* f. sp. *ciceris*, *Rhizoctonia bataticola*, and *Pythium* sp. fungi of chickpea (*Cicer arietinum* L.) in the rhizosphere of chickpea roots. Parameters of interest included size and time of the inoculum, survival, and competition for an ecological niche in what is described as non-sterile, fungal disease-conducive field soil (Nautiyal 1997a). Both *Pseudomonas* sp. NBRI9926 and *Rhizobium* NBRI9513 were tagged with Rif^r antibiotic resistance to allow for easier enumeration and recovery with minimal disturbance to the microbial fitness to assess the persistence of indigenous and nonindigenous microbes (van Elsas et al. 1986). Results showed aggressive colonization by *Pseudomonas* sp. NBRI9926 over the 45-day experiment with minimal activity being recorded by indigenous *Rhizobium* strain NBRI9513 suggesting that the isogenic or equally rhizosphere-competitive second non-isogenic *Pseudomonas* sp. NBRI9926 strain may preempt indigenous colonization of the soil when present in higher ratios (Nautiyal 1997a). Furthermore, greenhouse evaluations made by Nautiyal (1997a) concluded with the biological control of *Fusarium oxysporum* f. sp. *ciceris*, *Rhizoctonia bataticola*, and *Pythium* sp. by *Pseudomonas* sp. NBRI9926 and to a lesser extent *Rhizobium* NBRI9513. Similar results with sugar beets, potato, and wheat have also shown initial colonization of the roots by an equally competent and highly competitive strain, colonizing the developing root system throughout the season (Suslaw and Schroth 1982; Compeau et al. 1988; Kloepper et al. 1980).

Yield increases in sugar beet of 20–85 % as a result of phytopathogenic control by fluorescent *Pseudomonas* sp. have shown tremendous vigor in establishing rhizosphere competence and biological control (Compeau et al. 1988). Similarly, lettuce, tomato, and cucumber have also shown increases in root and fresh weight by *Pseudomonas* strains (van Peer and Shippers 1988). Fluorescent *Pseudomonas* sp. has been indicated in control of root rot in soybean, black root rot in tobacco, potato seed decay, several wilt diseases caused by *Fusarium* sp., fungal diseases of orange and lemon citrus roots, and cotton through inhibition of *Pythium ultimum*-infested soils, which resulted in a 71 % increase in seedling survival (Kloepper et al. 1980; Xu and Gross 1986; Yuen and Schroth 1986; Lifshitz et al. 1986; Becker and Cook 1988; van Peer and Shippers 1988; Keel et al. 1989). *Pseudomonas corrugata* has been found to increase the qualitative and quantitative yields for maize, one of the major cereal crops of India (Kumar et al. 2007). Similar competency acquiring strategies of screening, isolation of indigenous microorganisms, and selection of efficient strains were used under Himalayan temperate conditions.

Maize is known for producing consumable exudates containing different sugars, amino acids, and carbohydrates (Baudoin et al. 2001). In the first year experiments, *P. corrugata* increased in total yield of maize up to 144.9 %. Grain dry weight was also found to increase by 194.3 %, and the harvest index per unit was significantly higher over the control treatments. Third and fourth year experiments consistently

recorded similar results. Furthermore, an increase in total nitrogen and phosphorous content was seen with *P. corrugata*, reflecting the highest increase. At maturity, *P. corrugata* inoculated plants that resulted in 2.78 times higher root/shoot ratio over control plants. The length of the root and shoot for maize plants treated by *P. corrugata* was the most effective. The overall health of the plant was optimized due to various mechanisms of nitrogen fixation to the host plant, production of phytohormones, phosphate solubilization, and production of metabolites such as siderophores and antibiotics (Glick 1995; Whipps 2001; Compant et al. 2005). Treatment of maize with *P. corrugata* did not show any suppression of indigenous bacteria during the cultivation period (Kumar et al. 2007).

10.2.2 Chemotactic Responses During Competitive Colonization

Competition for nutrients supplied by root exudates exists as a primordial mode of survival, where beneficial rhizobacteria and phytopathogens excavate the rhizoplane for nutrients required for active metabolism and consequently for reproduction (Kalia and Mudhar 2011). *Pseudomonas* sp. has been regarded as the most effective root-colonizing group of soil microorganisms due to their nutrient-acquiring abilities, which prompt rapid root colonization and host-microbe feedback, indirectly and/or directly, initiating strategic defense mechanisms, which deprive and break down the invasive phytopathogen (Oksinska et al. 2011; Haas and Defago 2005). The versatile nature of *Pseudomonas fluorescens* as an effective root colonizer has been well documented with the strain expressing over 20 genes homologous to the genes involved in nutrient acquisition, stress response, and secretion (Bloemberg and Lugtenberg 2001).

Root exudates consist of organic compounds of low molecular weights such as sugars, amino acids, and organic acids which are present in the cytoplasm at high concentrations (Farrar et al. 2003). Chitinase, β -1, 3-glucanase, and protease enzymes have shown to combat limiting nutrient conditions by enabling *P. fluorescens* to release additional sources of nutrients through the degradation of fungal cell wall components and through protein hydrolysis, obtaining additional nitrogen, carbon, and energy sources important for colonization (Fridlender et al. 1993; O'Sullivan et al. 1991). However, changes in the composition of root exudates during root growth, due to environmental or ecological conditions, can have detrimental effects on the rhizobacterial composition and activity (Campbell et al. 1997). Such can be seen in environmental conditions depicting arid soils which facilitate porous deep layer reservoirs where nutrients and minerals percolate and thus become less attainable to the plant and rhizobacteria (Khan and Parmar 2013). Plant roots are generally drawn toward the upper and subsurface regions, elongating their root and arteries in means of attaining readily available surface nutrients and minerals. However, nutrients, minerals, and water often percolate through the soil pores toward a lower intermediate region. This area is often rich in resources and, however, difficult to attain due to the plant anatomy of the roots unable to harvest the length required to draw nutrients from nonlocalized regions.

As a result, as often seen in soils which are highly porous, evapotranspiration rates may dehydrate the plant, resulting in shutdown and formation of cysts (Khan and Parmar 2013; Singh et al. 2009). PGPR, such as *Pseudomonas* sp., are often brought in as stimulatory agents that promote root elongation and increased plant-nutrient feedback through their synergistic association. *Pseudomonas* sp. is an organism well established as a physiologically motile, O-antigen-producing and chemotactic organism, capable of successive integration in the numerous soil layer zones.

In Lugtenberg and Dekkers (1999) review of pseudomonads, severely impaired colonization was a result of *Pseudomonas* sp., mutants lacking motility, attributed to the impaired function of the flagella. The role of the flagella has been documented as a wild-type trait, existing in sand and in soil for the colonization of potato and tomato roots (de Weger et al. 1987; Simons et al. 1996; Dekkers et al. 1998). *Pseudomonas* spp. produce up to nine polar flagella per cell and are described as the wild-type successive colonizers. Nonmotile mutant *P. fluorescens* strain WCS374 was shown to be severely impaired in the colonization of potato roots, with the largest site of infections furthest from the site of inoculation (Lugtenberg et al. 2001a, b). Chemotactic characteristics of motile scavenging for root exudates were evaluated by Vermeiren et al. (unpublished), by isolating mutants defective in the general chemotaxis gene *cheA* of European *P. fluorescens* strains WCS365, OE283, SBW 25, and F113. Results showed a 100- to 1000-fold decline in the competitive root tip colonization of tomato by the nonmotile mutant strains. Interestingly, it was indicated that using wild-type *P. fluorescens* supplemented in a gnotobiotic system lacking *cheA* general chemotaxis gene did not stunt the ability of effective colonization of tomato, suggesting other factors are associated with the transportation and feedback mechanisms of the bacteria to the root tip (Geels and Schippers 1983; Scher et al. 1988; de Mot and Vanderleyden 1991; Rainey and Bailey 1996; Dekkers et al. 2000).

10.2.3 Physiological-Induced Attachment to Plant Roots

Establishment of plant-bacterium interaction involves a preliminary stage of attachment or infection of cell surface appendages mainly involving bacterial cells and the plant roots that result in nodulation (Lugtenberg and Dekkers 1999; Lugtenberg et al. 2001a, b). Pili and fimbriae have been reported in their role in the attachment of *P. fluorescens* 2-79 to corn roots (Vesper 1987). *P. aeruginosa* contain type 4 pili, which mediate the initial contact between the rhizobacteria and the epithelial cell surface through a locomotive mechanism known as twitching motility. This enables the bacteria to attach to abiotic surface and form biofilms (Pier 1985; Hahn 1997; Darzins and Russell 1997). Gene *pilA* is responsible for encoding prepilin, whereas pilus retraction and twitching motility is controlled by the *pilT* gene (Whitchurch et al. 1991). One to two type 4 pili were found in vitro attached on average per *P. fluorescens* WCS365 cell, while zero pili associated with mutant *pilA* gene and three to four pili associated on the surface of *pilT* mutant cells. In addition, expression of *pilA* promoter gene has been found associated in the tomato rhizosphere, while *pilA* mutant PCL1092 and *pilT* mutant PCL1093 were found ineffective in establishing competitive root tip colonization, suggesting that type 4 pilus

has an intermittent role in competitive tomato root tip colonization (Camacho Carvajal 2001). The roles of type 4 pili have also been well documented in their association as plant pathogens, considering them as virulence factors. However, due to their involvement in colonization of both plant and associative nitrogen-fixing bacterium (*Azoarcus*), more attempts to understand the role of pili are required (Dorr et al. 1998). Attachment of rhizobia to plant cells is regarded as the early step for plant-microbial interaction and the initial step for the formation of microbial biofilm on plant roots (Rodriguez-Navarro et al. 2007).

Various mechanisms and diverse surface molecules of rhizobia and host plants have been indicated to initiate the interaction. Plant lectins, found in more than 600 species of leguminous plants, account for up to 10 % of total protein content; are present in mature seeds, leaves, stems, and roots; and have been associated with binding by acting as receptors for bacterial surface polysaccharides (Rudiger and Gabius 2001; van Eijsden 1994; Hirsch 1999). Calcium ions (Ca^{2+}) which bind proteins called rhicadhesin have also been implicated in the bacterial attachment to legume root hairs, specifically by anchoring rhicadhesin to the rhizobial cell surface (Smit et al. 1991). These proteins have been described by binding calcium at the bacterial cell poles and to root hairs, initiating calcium-dependent agglutination (Gage 2004). Along with bacterial surface polysaccharides, legume lectins located at the root hair tip initiate binding through recognition of carbohydrate structures present in the bacterial surface and, however, are rather weak and reversible (Matthysse and Kijne 1998). These exopolysaccharides elicit infection thread formation in legumes forming intermittent nodules, as seen in alfalfa and clovers. In addition, roots inoculated with mutant exopolysaccharides *Rhizobium leguminosarum* bv. *viciae* had limited number of infection sites as compared to the wild type, suggesting that the exopolysaccharides are involved and could enhance bacterial binding to growing root hairs during the preliminary attachment phase (van Workum et al. 1998; Rodriguez-Navarro et al. 2007). Other studies describing the physiologically important characteristic of attachment through infection and nodulation have demonstrated active adsorption to roots by polymeric fibrillar bridges or through protein bridges (Albareda et al. 2006). Attachment mediated by cell surface proteins has been shown to involve with *Pseudomonas* spp. interaction with plant roots, specifically through the outer membrane protein OprF of *P. fluorescens* OE 28.3 strain (de Mot et al. 1992). Furthermore, the protein agglutinin, which mediates agglutination of bacterial cells to glycoprotein on the plant roots, has been isolated from *P. putida* strain Corvallis (Anderson et al. 1988). Agglutinin plays a major role in such adherence and colonization by *P. putida* strain Corvallis to bean and cucumber which has been regarded by Anderson et al. (1988) to be encoded by *aggA* locus in competitive colonization and adherence to roots with the parental strain on the roots of bean, potato, tomato, and grass (Glandorf 1992). However, 30 different *Pseudomonas* isolates on tomato, potato, and grasses were found to have no agglutination-dependent adherence, suggesting many other factors, molecules, and genes that are involved in the plant and bacteria attachment feedback mechanism (Glandorf 1992).

10.2.4 Chemical-Induced Mechanisms of Synthesis

The synergistic behavior of *Pseudomonas* spp. and other PGPR with plants and rhizobia has been well documented as a continuous systematic feedback mechanism, reverting to the bacterial community and colonization dependence on the nature and concentration of organic constituents of the host secreted exudates (Saharan and Nehra 2011). Chemical induction/uptake and catabolism of organic compounds through nitrogen fixation and phosphorus solubilization, among other factors, determine the relative associations and abilities of the bacteria to attach to the root surface appendages (rhizoplane) (Ashrafuzzaman et al. 2009; Vazquez et al. 2000). Improvement of soil fertility constitutes the enrichment of both readily available nitrogen and phosphorus to increase agricultural production, with nitrogen affixed to enhance soil fertility and phosphorus as a major essential macronutrient for biological growth and development, making insoluble soil inorganic phosphate available in soluble form for uptake by the host plant (Rodriguez et al. 2006; Chen et al. 2006). Due to the degree of importance, both nitrogen and phosphorus have been targeted for continuous improvement, traditionally as artificial chemical supplements and more recently through the use of biological entities for improved soil fitness and biological competence and adherence.

10.2.4.1 *Pseudomonas* spp. as Nitrogen Fixer

Nitrogen is a key component to many biomolecules, making up the two most important polymers of life, nucleic acids and proteins (Mia et al. 2013). Nitrogen exists primarily in the atmosphere as N_2 gas; however, fixed inorganic nitrogen ionic forms available as nitrate (NO_3^-) and ammonium (NH_4^+) are highly limiting in terrestrial and marine ecosystems, limiting crop growth and development (Mia and Shamsuddin 2010). Plants supply exudates to the nitrogen-fixing bacteria, convert nitrogen from air, and secrete part of the fixed nitrogen directly into the host cell as NH_4^+ for the plant use (Mia et al. 2013). In limiting conditions, root nodule symbiosis by nitrogen-fixing bacteria is unable to accommodate the legumes with enhancing capabilities to obtain fixed nitrogen (Quispel 1974). However, using diazotrophic bacteria, primarily bacteria, and archaea has shown success in attributing host plants capable of fixing their own nitrogen through close association with their synergistic diazotroph, although gains using *Pseudomonas* spp. for nitrogen fixation have been limited (Mia and Shamsuddin 2010). Among nitrogen-fixing bacteria, *Pseudomonas* sensu stricto were thought to be absent or inadequate and described as putative nitrogen fixers, later deemed to genera in the γ - and β -*Proteobacteria* (Chan et al. 1994; Young 1992). Consequently, in recent years, through analysis of physiological properties, nitrogenase assays, phylogenetic studies, and detection of *nifH* DNA, several strains of nitrogen fixers have been ambiguously identified as true *Pseudomonas* spp. (Chan et al. 1994; Desnoues et al. 2003). Two nitrogen-fixing bacteria are identified as *Pseudomonas stutzeri* strain CMT.9.A isolated from the roots of *Sorghum* (Desnoues et al. 2003) and *P. stutzeri* strain A15, isolated from paddy fields in China (You and Zhou 1989; You et al. 1991; Yan et al. 2008). *P. stutzeri* form a heterogeneous group comprising of seven to nine genomovars, reflecting a remarkable degree of physiological and genetic diversity

(Garcia-Valdes et al. 2003; Sikorski et al. 1999). Strain A15 is widely used in inoculating rice in China, which is biologically regarded as an endophyte of rice. This is in agreement to the A15, also known as the A1501, which is promoting plant growth (Vermeiren et al. 1999).

Desnoues et al. (2003) evaluated similarities between *P. stutzeri* strain A1501 and the *nif* and *rnf* genes involved in the electron transport to nitrogenase in *Rhodobacter capsulatus*, which ran parallel to the arrangement of 3 genes involved in the regulation of nitrogen fixation process in the same order as *Azotobacter vinelandii*. Systemic mutagenesis of the *nif* and *rnf* genes confirmed the lack of nitrogen fixation, thus establishing concrete evidence that *nif* and *rnf* in strain A1501 are essential for nitrogen fixation. Furthermore, by using *lacZ* fusions, *nif* and *rnf* gene expression were found to be regulated by the *ntrBC*, *nifLA*, and *rpoN* genes, whereas *rnf* gene expression was a direct precursor for the regulation of the nitrogen fixation process (Dixon 1998; Fischer 1994). Advancement of research *P. stutzeri* family of nitrogen fixers revealed *P. stutzeri* DSM4166 which is analogous to strain CMT.9.A and is isolated from the rhizosphere of *Sorghastrum nutans* cultivar in Germany (Krotkzy and Werner 1987; Yu et al. 2011). Strain DSM4166 revealed a genome similar in size, with a 66.8 % correspondence with strain A1501 and contained a *nif* island with similar gene organization (Yan et al. 2008; Yu et al. 2011). *P. stutzeri* strain 6H33b is another strain isolated from the *Pseudomonas* spp. that has shown nitrogen-fixing abilities (Hatayama et al. 2005). Isolated from a compost pile in Japan, nitrogen-fixing *nifH* and *nifD* genes showed close relation to the nitrogen-fixing genes of *P. stutzeri* strain A1501 and taxonomic similarity to *P. indica* strain 6H33b. However, this could be differentiated with its distinguishable nitrogen-fixing ability and absence of nitrate reduction, denitrification, and utilization of some sugars and organic acids, which are prominent features in several non-nitrogen-fixing *Pseudomonas* sp. Strain 6H33b was later classified as *Pseudomonas azotifigens* sp. nov. (Hatayama et al. 2005; Bergersen 1991).

10.2.4.2 *Pseudomonas* spp. as a Phosphate-Solubilizing Bacteria

Phosphorus is the second most important and expensive nutrient source required in the growth and development of plants and organisms. Phosphorus availability in soil is markedly low or unavailable and found only in the form of insoluble phosphates which cannot be utilized by plants (Alam et al. 2002). Majority of the soils around the world are deficient in phosphorus with consumable phosphorus accounting to only 1–5 % available for plants (Molla and Chaudhury 1984). Availability is limited due to the rapid fixation/sorption of phosphorus, resulting in the production of oxides and hydroxides of calcium, aluminum, and iron (Rajankar et al. 2007; Richa et al. 2007; Deepa et al. 2010; Tao et al. 2008). To counteract such limited availability, microbial communities capable of transforming insoluble/bound phosphorus into soluble form are termed accordingly as phosphate-solubilizing bacteria (PSB) (Khan et al. 2013; Yadav et al. 2011). Applications of such PSB have shown tremendous gains, where most PSB are acknowledged by the *Pseudomonas* genera (Behbahani 2010; Ahemad and Khan 2011) *Bacilli*, *Rhizobia*, and *Azotobacter* (Wani et al. 2007; Yadav et al. 2011; Abd-Alla 1994; Chandra et al. 2007; Marra et al. 2011; Yi et al. 2008).

Pseudomonas fluorescens and *P. aeruginosa* are characterized as PSB with the ability to produce secondary metabolites such as cyanide, which is regarded as ecologically important, giving a selective advantage to the PSB by managing various plant diseases caused by phytopathogens and indirectly promoting growth (Siddiqui et al. 2006; Vassilev et al. 2006; Badawi et al. 2011). *P. fluorescens* enhanced germination of groundnut plants (*Arachis hypogaea*) by 30 % while increasing grain yield by 77 %. Furthermore, to understand the biocontrol potential, *Macrophomina phaseolina*, a plant pathogen that was added, resulted in a 57 % decrease in grain yield, suggesting not only was *P. fluorescens* solubilizing phosphorus to enhance grain yield, it was also acting to protect the plant against *M. phaseolina* (Shweta et al. 2008; Khan et al. 2013). *P. fluorescens* was also evaluated for PSB characteristics to enhance peanut plant growth. Observations were described with increased pod yields up to 26 %, increased haulm yield, nodule dry weight, and higher nitrogen and phosphorus content in soil. Furthermore, physical characteristics of root length, pod number, 100-kernel mass, shelling outturns, and number of nodules also increased. Interestingly, incidence of collar rot and charcoal rot, a soilborne fungal disease caused by *A. niger*, decreased through inoculation of PS *P. fluorescens*, attributing the secondary control activity to be regulated by other plant growth-promoting substances such as the synthesis of IAA, ACC deaminase, siderophores, and antifungal compounds (Dey et al. 2004). *Pseudomonas* spp. and other PGPR can share dual inoculation as they act as synergistic inoculants in aims to increase the fitness of a plant (Khan et al. 2013). A composite application of N₂-fixing *S. meliloti* and PSB, *Bacillus* sp.M7c and *Pseudomonas* spp. FM7d significantly enhanced the N-fixing ability of alfalfa plants (*Medicago sativa* L.) In this treatment, *Pseudomonas* sp. FM7d was attributed to enhance dry matter of roots and shoots, increased length and surface areas of roots and, consequently, the increased symbiotic associations with alfalfa plants (Guinazu et al. 2010). Increases in cereal production following the inoculation of PSB, *P. fluorescens* 153, *P. fluorescens* 169, *P. putida* 4, and *P. putida* 108, have also been reported as a result of phosphate-solubilizing activity synthesizing growth-promoting factors as mentioned earlier. Increases in phosphate uptake, accounting to 80–96 %, and wheat grain yield, 37–58 %, under both field and greenhouse conditions were seen when inoculated with *P. putida* 108 (Zabiha et al. 2011).

Pseudomonas sp. CDB35 application in maize (*Zea mays*) was also studied. Inoculated in the rhizosphere of maize, Hameeda et al. (2008) observed enhancement of dry matter with a 94 %, increased grain yield of 64 %, and overall biomass of maize. When applied as a dual inoculant with arbuscular mycorrhizal (AM) fungi *Glomus intaradices*, *P. fluorescens* continued to increase plant growth, nutrient uptake grain, and other yield components, such as harvest index, grain nitrogen and phosphorus content, readily available soil phosphorus, and root colonization percentages under water-stressed conditions (Ehteshami et al. 2007; Hameeda et al. 2008). As a single inoculant, however, *P. fluorescens* production was less in numbers, suggesting the role of AM fungi attributing to the enhanced activity, indicative to its function as a suitable symbiotic bioinoculant (Khan and Parmar 2013; Zaidi et al. 2001).

Pseudomonas spp. such as *P. striata*, *P. aeruginosa*, *P. putida*, *P. vermiculosam*, and *P. fluorescens*, are involved as PSB; however, factors such as carbon and nitrogen, mineral source, pH, incubation temperature, incubation period, and aeration have been regarded (Kumar et al. 2002; Vassilev and Vassileva 2003; Taiwo and Oguundiya 2008). Ecologically, this creates an environment where rhizobia are continuously adapting and interacting with the beneficial PSB, such as *Pseudomonas* spp., fixing nitrogen and readily exchanging nutrient and minerals through the plants vascular system back into the soil. As a result, competition becomes highly regulated, as the PGPR can develop regional dominance within the system and actively coordinate activities between the host plant and indigenous microflora without inducing invasive response (Glick 1995). With the appropriate inoculations and combinations, PSB can increase crop yields up to 70 %, attributing to the enhanced phosphorus solubilization, which in turn instigates plant growth promotion through improvements in biological nitrogen fixation (Kucey et al. 1989; Verma 1993).

10.3 Biologically Induced Mechanisms of Resistance

Biologically induced mechanisms of phytopathogenic control using *Pseudomonas* spp. have been well documented with many of the findings showing strong correlation between plant growth promotion and the intrinsic expression of siderophores, HCN, and antibiotic concentrations, as active metabolites within the rhizosphere (Siddiqui et al. 2006; Oves et al. 2009). These mechanisms of biocontrol induce the production and release of growth factors, which in turn mediate plant-host feedback by developing a module for systematic communication to establish rhizosphere competency, colonization, and rhizobial interactions (Saharan and Nehra 2011).

An assortment of *Pseudomonas* spp. has been identified for their role in biological suppression of phytopathogens, such as *P. fluorescens*, *P. putida* (Zabiha et al. 2011), *P. aeruginosa* (Ahemad and Khan 2010), fluorescent *Pseudomonas* (Shweta et al. 2008), *P. vancouverensis* (Mishra et al. 2008), *P. jessenii* (Rajkumar and Freitas 2008), and *Pseudomonas* sp. (Poonguzhali et al. 2008). At present, several commercial products using *Pseudomonas* spp. are on the market, of which, *P. aeruginosa* PSS production of antimicrobial metabolites, siderophore pyoverdine and salicylic acid (SA), has shown to be highly effective against *Peronospora tabacina* in tobacco culture, *Alternaria solani* in tomato, and *Pseudoperonospora cubensis* in cucumber (Diaz de Villegas 2007). Fluorescent *Pseudomonas* spp. have also shown tremendous success in the effective suppression of many soilborne plant diseases due to their capacity to produce a wide variety of antibiotics, chitinolytic enzymes, growth-promoting hormones, siderophores, HCN, catalase, and regarded as a highly effective PSM (Bagnasco et al. 1998; Kraus and Loper 1995; Rodriguez and Fraga 1999; Seong and Shin 1996). We will further examine the role and mechanism by which *Pseudomonas* spp. biologically control phytopathogens. Important strains of *Pseudomonas* showing antibiosis against fungal plant pathogens are listed in Table 10.1.

Table 10.1 Important *Pseudomonas* strains showing biocontrol activity against plant pathogens

Sr. no.	Strain	Effector molecule/metabolite	Mode of action	Target pathogen	Host plant origin	References
1.	<i>P. aurantiaca</i>	DAPG	Antibiosis	<i>F. oxysporum</i>	Wheat	Keel et al. (1989)
2.	<i>P. aureofaciens</i>	PCA (phenazine-1-carboxylic acid)	Antibiosis	<i>G. graminis</i> var. <i>tritici</i>	Wheat	Thomashow and Weller (1988)
3.	<i>P. aeruginosa</i>	Anthranilic acid	Inhibition of growth	<i>F. oxysporum</i> f. sp. <i>ciceris</i>	Chickpea, pigeon pea	Anjiah et al. (1998)
4.	<i>P. cepacia</i>	Pyocyanin	Antibiosis	<i>Septoria tritici</i>	Wheat	Baron et al. (1997)
		Pyrrolnitrin	Antibiosis	<i>Aphanomyces cochlioides</i>	Sugar beet	Hill et al. (1994)
5.	<i>P. chlororaphis</i> 30–84	Phenazines, pyrrolnitrin, antibiotics HCN, pyoverdine	Antibiosis	<i>G. graminis</i> var. <i>tritici</i>	Wheat	Pierson and Pierson (2010), Loper et al. (2012)
	06	Phenazines, pyrrolnitrin, HCN, pyoverdine	Antibiosis, ISR	<i>Phytophthora</i> , <i>Corynespora</i> , <i>Pectobacterium</i>	Vegetable crops; potato	De Vleeschauer and Höfte (2009), Park et al. (2011)
	PCL 1391	Phenazine, HCN (phenazine-1-carboxamide), pyoverdine	Antibiosis	<i>Fusarium</i>	Chickpea	Chin-A-Woeng et al. 2001; Ruffner et al. 2013.
6.	<i>P. fluorescens</i>					
	DR 54	Viscosinamide, chitinase pyoverdine	Antibiosis	<i>P. ultimum</i> <i>R. solani</i>	Sugar beet	Nielsen and Sørensen (2003), Sanguin et al. (2008)
	F113	DAPG, HCN, pyoverdine ACC deaminase, T3SS	Antibiosis	<i>P. ultimum</i> , <i>R. solani</i> <i>P. carotovorum</i>	Sugar beet Potato	Redondo-Nieto et al. (2013)
	KD	T3SS, HCN, pyoverdine	Antibiosis	<i>P. ultimum</i> <i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	Cucumber tomato	Rezzonico et al. (2005)

(continued)

Table 10.1 (continued)

Sr. no.	Strain	Effector molecule/metabolite	Mode of action	Target pathogen	Host plant origin	References
	Q2.87	DARG, HCN, ACC deaminase pyoverdine	Antibiosis, ISR	<i>G. graminis</i> var. <i>tritici</i>	Wheat	Weller (2007), Weller et al. (2012), Loper et al. (2012)
	SBW 25	Viscosin, pyoverdine T3SS, competition	ND	<i>P. ultimum</i>	Pea	Sanguin et al. (2008), Trippe et al. (2013)
	SS101	Massetolide, pyoverdine	ISR	<i>P. ultimum</i> , <i>P. infestans</i> , <i>P. syringae</i> pv. <i>tomato</i>	Cotton, tomato	Loper et al. (2012), van deMortel et al. (2012)
	WCS374	Pyoverdine, pseudomonine, salicylate, T3SS	ISR	<i>F. oxysporum</i> f. sp. <i>raphani</i> , <i>Magnaporthe poae</i> <i>P. syringae</i> pv. <i>tomato</i>	Cotton, chilies Tomato	Pieterse et al. (2003), Bakker et al. (2007), De Vleeschauer and Höfte (2009)
	WCS417	–	ISR	<i>Fusarium</i> , <i>Alternaria</i> , <i>Hyaloperonospora</i>	Vegetable crops	Bakker et al. (2007), VanderEnt et al. (2008)
	1P24	DAPG, HCN, pyoverdine	Antibiosis	<i>R. solanacearum</i> <i>G. graminis</i> var. <i>tritici</i> <i>R. solani</i>	Tomato Wheat Cotton	Sanguin et al. (2008)
	2-79	Phenazine, pyoverdine (phenazine-1-carboxylic acid)	Antibiosis	<i>R. solanacearum</i> <i>G. graminis</i> var. <i>tritici</i> <i>R. solani</i> <i>Magnaporthe poae</i>	Wheat Kentucky Blue grass	Cook et al. (1995) Weller (2007) Mavrodi et al. (2006)

<i>P. protegens</i>	CHAO	DAPG, HCN, pyoluteorin, pyoverdine, salicylate, pyrrolnitrin	Antibiosis ISR	<i>Thielaviopsis basicola</i> <i>G. graminis</i> var. <i>tritici</i> <i>R. solani</i> , <i>P. ultimum</i>	Tobacco, Wheat Cucumber	Haas and Defago (2005)
	Pf-5	DAPG, pyrrolnitrin, HCN, pyoluteorin, rhizoxin, orfamide, pyoverdine, enantiopyochelin	Antibiosis, ISR	<i>P. ultimum</i> , <i>R. solani</i> <i>Drechslera poae</i> <i>Sclerotinia homeocarpa</i>	Cotton, Cucumber, Blue grass	Gross and Loper (2009), Loper et al. (2012), Weller et al. (2012)

10.3.1 Phytopathogen Suppression by Siderophores

Siderophores are described as iron carriers or iron-binding compounds that are water soluble, low molecular weights, and organic ligands with affinity and specific iron-binding capacity (Kraemer 2004). Iron is regarded as the fourth most abundant element in the Earth's crust; however, under aerobic conditions, free ferrous iron (Fe(II)) becomes oxidized to ferric iron (Fe(III)), which is highly insoluble making iron unavailable for metabolic use by living organisms (Neilands 1995; Fernandez Scavino and Pedraza 2013). It has been found that most siderophores belong to the gram-negative isolates, *Pseudomonas* and *Enterobacter* genera (Tian et al. 2009). Fluorescent siderophores belonging to the fluorescent pseudomonad species have shown to demonstrate considerable genetic and biochemical evidence toward the biological control of soilborne plant pathogens in disease suppressive soils (Viswanathan and Samiyappan 2007).

To enhance the level of iron uptake in the rhizosphere, *Pseudomonas* sp. possesses the ability to utilize heterologous siderophores produced by diverse species of bacteria and fungi, with *P. putida* showing enhanced iron requisition through the use of siderophores produced by *Rhizobium* sp. (Suttiviriya et al. 2008). Production of siderophores that chelate and consequently scavenge ferric iron in the rhizosphere may prompt the direct inhibition of other microorganisms whose affinity for iron is less (Kloepper et al. 1988). Furthermore, by binding Fe(III) in the rhizosphere, proliferation of fungal phytopathogens and direct competition become limited, where the release of specific siderophores favors the root-colonizing ability of the biocontrol strain (Aeron et al. 2011). Using maize seeds inoculated in iron-stressed conditions with siderophore producing fluorescent *Pseudomonas* spp. strains GRP3A and PRS, Sharma and Johri (2003) reported significant increase in germination percentage and plant growth. With as little as 10 μ M Fe(III) and an assortment of native bacterial inoculants, maximum shoot and root length and dry weights were observed (Sharma and Johri 2003). Pandey et al. (2005) observed inoculation with *P. aeruginosa* GRC1 strain which is capable of producing hydroxamate siderophores, similar to pyoverdine, in iron-deficient conditions while enhancing the growth of *Brassica campestris* var. Pusa Gold (Indian mustard). Moënne-Loccoz et al. (1996) studied whether increasing the spectrum of siderophores could improve the ecological competence in the rhizosphere of sugar beet using *Pseudomonas* sp. B24Rif(pCUP2), with plasmid pCUP2 carrying a copy of the *pbuA* gene, serves as a membrane receptor for Ps114 to complement *Pseudomonas* sp. B24Rif strains' own ferric siderophores. This allowed the B24Rif (pCUP2) strain to utilize ferric siderophores from a larger fraction of resident fluorescent pseudomonads, suggesting the resident community of fluorescent *Pseudomonas* spp. also possessed the Ps114 like siderophore.

Similarly, competitive and colonizing behavior of *P. fluorescens* WCS374 in the rhizosphere of radish was studied. Ferric-siderophore transport into the bacterial cell commences by binding the ferric-siderophore complex to specific receptor proteins, whereby the level of expression is regulated through the concentration of available iron (Neilands 1982). Many genes encoding ferric pseudobactin

receptors of fluorescent pseudomonads such as PupA have been sequenced and cloned to promote binding to different strains of *Pseudomonas* spp. (Bitter et al. 1991; Raaijmakers et al. 1995). Siderophore receptor PupA for ferric pseudobactin358 was introduced via a cosmid pMR into *P. fluorescens* WCS374, with the resulting WCS374(pMR) found to be more competitive than the wild-type WCS374 strain when co-inoculated with *P. putida* WCS358, which produces pseudobactin (Raaijmakers et al. 1995). In addition to the pseudobactin, fluorescent pyoverdine siderophores directly complex to ferric iron in the soil or root zone as a result of their high affinity which are taken up via outer membrane receptors and synthesized accordingly, whereas low-affinity siderophores of phytopathogens are left dormant and with minimal to no iron uptake, resulting in starvation and demise, thus reflecting biological dominance (Lemanceau et al. 1992a, b).

Fluorescent siderophore pyoverdine has been indicated for its role in plant growth stimulation (Hofte et al. 1991). This mode of biological resistance was shown earlier by Vandenbergh and Gonzalez (1984) using a mutant *P. putida* strain NRRL-B-12537 that overproduced siderophore molecules in the rhizosphere of tomato plants, showing pathogen resistance against *F. oxysporum*. Results revealed a highly effective mutant strain, suppressing *F. oxysporum* activity more so than the wild-type *P. putida* strain. Similarly, a mutant *P. aeruginosa* strain, defective in its ability to produce siderophore molecules, was tested for its intrinsic level of biocontrol against *Pythium* sp. in tomato plants. Results confirmed *Pythium* infection in tomato plants, as parameters marketing iron consumption were solely induced by *Pythium* sp., rendering mutant *P. aeruginosa* siderophore complex inactive (Buysens et al. 1994; Khan and Parmar 2013). *Pseudomonas* sp. WCS417r strain has shown resistance to *Fusarium* wilt on carnation caused by *Fusarium oxysporum* f. sp. *dianthi* (Fod). Duijff et al. (1993) used mutant WCS417r strain, defective in its capacity for siderophore biosynthesis (sid^-), and compared it to *Pseudomonas putida* strain WCS358r. The team inhibited conidial germination by purified pseudobactins, which are siderophore molecules of *Pseudomonas* species, and found the ferrated psuedobactins inhibited germination significantly less than the unferrated pseudobactins. Furthermore sid^- mutant WCS358 was ineffective in inhibiting Fod, whereas sid^- WCS417r was still able to inhibit Fod. Treatment with WCS358r strain on carnation was able to reduce *Fusarium* wilt, suggesting inhibition of Fod was induced solely on siderophore-mediated competition for iron. WCS417r strain significantly reduced incidence, while mutant sid^- WCS417r strain showed intermediate effectiveness in reducing wilt, suggesting WCS417 mechanism of pathogen resistance extends beyond siderophore inhibition, involving multiple mechanisms of control (Duijff et al. 1993). In addition, production of pyoverdine and salicylic acid (SA) by *P. fluorescens* CHAO suppressed necrosis caused by tobacco necrosis virus (Meyer et al. 1992; Maurhofer et al. 1994). Such mechanism of siderophore integration has shown to sustain synthesis even at low Fe^{3+} concentrations as plants are independent of the physical uptake process and, however, dependent on siderophore uptake and consequential release into the plant cellular components (Crowley et al. 1988; Wang et al. 1993).

Sugarcane cultivation in Indian soils is characterized typically by soils with high pH and lacking iron availability for plants (Rakkiyappan and Thangavelu 2000). As a result, red rot disease caused by phytopathogen, *Colletotrichum falcatum*, is common (Viswanathan and Samiyappan 1999). The production of siderophores by different strains of *Pseudomonas* spp. can act antagonistically against *C. falcatum* by chelating essential nutrients away from the soil to the plants, producing secondary metabolites and antibiotics such as IAA, HCN, and 2,4-DAPG, thereby directly antagonizing growth and activating ISR against the pathogen (Conrath et al. 2002; Viswanathan and Samiyappan 2004). Under iron-limiting conditions, Viswanathan and Samiyappan (2007) further witnessed *C. falcatum* mycelia growth suppression by antifungal siderophores produced at varying levels for all the *Pseudomonas* strains subjected for their investigation. As a result, the potential to antagonize certain soilborne pathogens at the saprophytic stage of growth may be possible through manipulation of available iron concentrations in the rhizosphere.

10.3.2 Phytopathogen Suppression by Antibiotics

Biological control of phytopathogens and undesirable microorganisms have been widely affixed to the phenomena of antibiosis and secondary metabolites that impede the growth life cycle of the pathogen, leading to its demise (Kalia and Mudhar 2011). Antibiosis by antibiotics and bacteriocins has been recognized as the most common mechanism of biocontrol, with fluorescent pseudomonads showing unique system of antibiosis against root pathogens like *P. fluorescens* which produce a variety of antibiotics on roots grown in soil. Particularly, it is the phenazine derivatives that are active against the take-all disease in wheat along with pyrrolnitrin, 2,4-diacetylphloroglucinol (DAPG), and pyoluteorin which have been frequently identified as classes of antibiotics produced by *Pseudomonas* spp. (Nielsen et al. 1999). *Pseudomonas fluorescens* strain CHA0 produces 2,4-DAPG and pyoluteorin, which has been shown to interfere with the growth of numerous pathogens and readily prompt disease suppression while exhibiting a quantitative relationship between the level of antibiotic production and the degree of suppression (Maurhofer et al. 1994; Kalia and Mudhar 2011). DAPG and pyoluteorin are antibiotics classified as nonvolatile polyketides produced by *P. fluorescens* capable of a broad spectrum of action against pathogenic fungi, bacteria, and nematodes (Haas and Keel 2003).

Active suppression through antibiotic gene expression by *P. fluorescens* involves relaying signal molecules such as *N*-acyl-homoserine lactones (AHL) (Pierson et al. 1998). Maurhofer et al. (1994) engineered a wild-type *Pseudomonas fluorescens* CHA0 strain to overproduce pyoluteorin and DAPG to test the relative resistance of disease caused by *Pythium ultimum* in cucumber plants. With similar finding to Schneider et al. (1994), an increase in the synthesis of antibiotics by a mutant strain of *P. fluorescens* CHA0 resulted in more aggressive suppression of *Pythium ultimum* compared to the wild-type *P. fluorescens* CHA0 (Maurhofer

et al. 1994). *Pseudomonas fluorescens* in particular have been recognized for their role in the suppression of the take-all disease, *Gaeumannomyces graminis* var. *tritici*, in wheat through the production of phenazine-1-carboxylic acid (PCA) and DAPG, accounting for 50–90 % take-all suppression (Cook et al. 1995; Weller 2007). DAPG has also been associated with the suppression of black root rot of tobacco plants by *Pseudomonas* spp. due to their high concentration present often in tobacco rhizosphere (Ramette et al. 2006). *Pseudomonas fluorescens* (Migula) F113 has been shown to control the soft rot of potato pathogen, *Erwinia carotovora* subspecies *atroseptica*, by DAPG production (Whipps 2001). Similarly, treatment of *P. fluorescens* against nematode *Globodera rostochiensis* saw a decrease in the emergence of nematode cysts and reduced juvenile mobility (Cronin et al. 1997). In context, three *P. fluorescens* strains UP61, UP143, and UP148 were evaluated on Uruguayan soils to confer the level of resistance of bird's-foot trefoil (*Lotus corniculatus*) from infection caused by *Pythium ultimum* and *Rhizoctonia solani* in vivo (Bagnasco et al. 1998). It was found that *P. fluorescens* had produced DAPG, pyoluteorin, and pyrrolnitrin (De La Fuente et al. 2004), while an antifungal phenazine derivative was produced by *P. fluorescens* strain UP148 (Bajsa et al. 2005). Traces of HCN and siderophores were also detected during the suppression of *Pythium ultimum* and *Rhizoctonia solani* (Bagnasco et al. 1998). *Pseudomonas fluorescens* strain NBRI1303 was identified as a single biocontrol antagonist toward *R. bataticola*, *F. oxysporum* f. sp. *ciceris*, and *Pythium* sp., the three most devastating pathogenic fungi of chickpea (Nautiyal 1997a) (Table 10.1).

Rifampicin-resistant mutant *P. fluorescens* strain NBRI1303R confirmed its capacity to control pathogen infection by rapid and aggressive root colonization. In particular, strain NBRI1303 reduced the incidence of diseased plants by 45 %, as well as significantly promoted germination, yield, length, and overall biomass of chickpea harvest (Nautiyal 1997b). Biocontrol ultimately is initiated by DAPG prompting its own biosynthesis and acting as a diffusible signal for increasing the synthesis and expression of DAPG biosynthetic genes (Maurhofer et al. 2004). A complex known as the GacS/GacA system controls DAPG (Phl) synthesis at the transcriptional and posttranscriptional level and facilitates active response to changes in gene expression and sensory signals. AHL in most *Pseudomonas* sp. is recognized as exerting a positive impact on cell densities. Upon activation, GacS/GacA modulates expression of exoenzymes, antibiotics, and HCN during cellular transition from exponential to stationary phases of growth, mandating subsequent interactions and competency in the rhizosphere (Fuqua et al. 1994; Sacherer et al. 1994; Heeb and Haas 2001; Khan and Parmar 2013). Environmentally responsive GacS/GacA regulatory system is of key importance in synthesis of Phl by *P. fluorescens*. Genetic modifications of the transcriptional regulatory control result in overproduction of Phl. Such mutations have been found useful for development of more potent biocontrol agents (Fenton et al. 1992; Delany et al. 2001). Other extracellular signals influencing metabolite synthesis can be suitably modified to enhance production of antifungal metabolites (Abbas et al. 2004).

10.3.3 Phytopathogen Suppression by Phenazines

Phenazines are nitrogen-containing heterocyclic molecules with broad-spectrum antibiotic properties. Evidence has shown that phenazines may be responsible for the ecological competence and the biological activity of pseudomonads (Mazolla et al. 1992; Turner and Messenger 1986). Both pathogenic and beneficial pseudomonads produce phenazines, *Pseudomonas aeruginosa*, a soilborne and opportunistic pathogen that produces several phenazines including pyocyanin which is closely related to pathogenicity. In contrast the root colonizers, *Pseudomonas fluorescens* and *P. chlororaphis*, produce phenazines which are responsible for suppression of fungal diseases of plants (Thomashow and Weller 1988; Pierson and Pierson 1996). Several mechanisms of inhibition have been proposed including inhibition of DNA replication, uncoupling of electron transport and energy production, as well as disruption of normal membrane functions (Pierson and Pierson 1996). Phenazine gene (*phz*) expression has been reported to influence the developmental stage of the root and responds to the presence of exogenous nutrients (Gong and Pierson, unpublished; Pierson and Pierson 1996). *Pseudomonas aeruginosa* PNA1, *P. chlororaphis* PCL1391, and *P. fluorescens* 2-79 have been widely recognized for their biological activity against a number of phytopathogenic fungi and oomycetes through the biosynthesis of antibiotic derivatives such as phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), and pyocyanin (Chin-A-Woeng et al. 1998; Anjaiah et al. 1998). *Pseudomonas aeruginosa* PNA1 isolated from the rhizosphere of chickpea has been known to exhibit biocontrol of phytopathogenic fungi such as *Fusarium* spp. on chickpea and pigeon pea, against *Pythium splendens* on bean and *Pythium myriotylum* on cocoyam (Anjaiah et al. 1998, 2003; Tambong and Hofte 2001). *Pseudomonas fluorescens* 2-79 mutant strains with the inability to produce PCA provided significantly less protection against take-all disease on wheat seedlings (Thomashow and Weller 1988). Similarly, *P. chlororaphis* strains impaired in the ability to produce PCN were unable to suppress disease formation in a tomato foot and root rot system (Chin-A-Woeng et al. 1998).

Little is known about the regulation and mechanism of action of phenazines in antifungal interactions; however, a basic understanding of the genetic interactions and signaling mechanism for cell density-dependent regulation of phenazines has been revealed (Chin-A-Woeng et al. 2003; Pierson and Pierson 1996). Phenazine gene (*phz*) expression in *P. aureofaciens* is regulated as a response to the environment, triggering two genes, *phzR* and *phzI*, to solicit activation. The *phzR* encodes a transcriptional activator that induces *phz* expression in response to *phzI* production, while the *phzI* gene is responsible for the production of a diffusible signal that induces phenazine production (Dunlap 1996; Pierson et al. 1995; Wood and Pierson 1996). Schippers and Gams (1979) had found that the spread of fungal infection through the root of the plant is reduced, along with the growth of external *G. graminis* var. *tritici*, runner hyphae as a result, increasing exudate release from lesion sites. This ultimately causes *P. aureofaciens* populations to increase, prompting elevated intracellular signal accumulation and activating phenazine

production at high levels, resulting in inhibition of growth of the pathogen. Biocontrol activity of nonmotile Tn5 transposon mutants belonging to the *P. chlororaphis* PCL1391 strain was found to be impaired by at least 1000-fold in its ability to protect tomato root tip invasion by *Fusarium oxysporum* f. sp. *radicis-lycopersici* when compared to the wild-type strain (Chin-A-Woeng et al. 2000). Furthermore, Anjaiah et al. (1998) found that mutants of *P. aeruginosa* PNA1 impaired in the transposon regions were unable to produce phenazine and as a result were unable to provide biological control against *Fusarium* spp. and *Pythium* spp. Several strains of *Pseudomonas* are already being marketed as commercial biocontrol agents because of their biocontrol abilities as seed treatment against seed-borne diseases in barley and oats based on the phenazine production by *P. chlororaphis* (Chin-A-Woeng et al. 2003). The commercially used *Pseudomonas* strains as biocontrol agents are referred as biopesticides and are regulated by the Environmental Protection Agency (EPA), USA. Biopesticides are promoted as part of the integrated pest management (IPM) approach. Utilization of the *Pseudomonas* inoculants in Europe is also regulated and requires approval under the directive (91/416/EEC) of the European Food Safety Authority (EFSA). In India, the Central Insecticide Board and Registration Committee under the Ministry of Agriculture regulates the production and marketing of biopesticides under section 9(3) of the Insecticide Act, 1968. *Pseudomonas fluorescens* figures prominently among the registered microorganisms and their products as bioinsecticides. There are over sixty companies which produce and market *Pseudomonas fluorescens*-based products as bioinoculants for control of fungal pathogens of the total 150 companies registered for production of bioinsecticides in India. Some of the *Pseudomonas* products being marketed are listed in Table 10.2.

10.3.4 Phytopathogen Suppression Through Induced Systemic Resistance

Induced systemic resistance (ISR) is another mechanism of providing biological resistance from phytopathogens through systemic expression of underlying signal pathways that elicit strong periods of resistance against fungi, bacteria, and viruses (Heil and Bostock 2002). Localized infection results in plants secreting a salicylic-dependent signaling cascade, as SA synthesis indicates infectious response, causing endogenous levels of SA to increase locally and systemically, as well as an increase in the plant phloem (Saharan and Nehra 2011). ISR was best described through the resistance against the tomato mosaic virus (TMV), reporting a reduction of weight up to 59 % with a mean disease incidence recording at 55.98 % (Kirankumar et al. 2008; Cherian and Muniyappa 1998). Several of the pseudomonad PGPR isolates were able to control early blight disease of tomato caused by *Alternaria solani* through induced system resistance (Earnapalli et al. 2006; Khan and Parmar 2013). *Pseudomonas fluorescens* ENPF1 and *P. chlororaphis* isolates have shown to promote plant growth while inducing systemic resistance against stem blight pathogen *Corynespora cassiicola* of *Phyllanthus amarus* (Msthiyazhagan

Table 10.2 Commercially available *Pseudomonas*-based biocontrol products

Product name	Biocontrol	Target pathogen	Crop	Manufacturer
Biofect Spot	<i>Pseudomonas aureofaciens</i> Tx1	Sclerotinia homeocarpa (Dolles Spot); <i>Pythium aphanidermatum</i> , (Colletotrichum graminicola anthracnose)	Turf	Eco soil system Inc., San Diego (CA)
Bio-save 10LP	<i>P. syringae</i> ESC-10 ESC-11	<i>Botrytis cinerea</i> , <i>Penicillium</i> spp. <i>Mucor piriformis</i> <i>Geotrichum candidum</i>	Pome fruit citrus, Cheshire, potatoes	Eco Science Corp., Longwood (Lyophilized product, frozen cells suspended in water applied as postharvest to fruit as drench/drop/spray)
Blight Ban A506	<i>P. fluorescens</i> A506	<i>Erwinia amylovora</i> and resist inducing bacteria	Almond, apple, apricot, blueberry, cherry, peach, pear, strawberry, and tomato	Nufarm Inc (Burr Ridge) (mettable powder spray at blooming)
Cedemon	<i>P. chlororaphis</i>	Leaf stripe, net block, <i>Fusarium</i> sp., spot block, leaf spot	Barley, oats	BioApri, Upsala (seed treatment)
Frost Bom	<i>P. fluorescens</i> A506, 1629RS <i>P. syringae</i> 742RS	Frost-forming bacteria	Fruit crops, almonds, potato, and tomato	Frost technology crop. Plant Health Technologies (Lathrop), CA
Bactvipe Phasal Rakshak	<i>Pseudomonas fluorescens</i>	Soilborne and foliar fungal pathogens	Wheat, chickpea, pigeon pea, mustard apple, tea, and vegetables	International Peuvera protech. haridwar, Uttrakhand (Seed treatment Foliar spray)
Floreghzen-P	<i>P. fluorescens</i>	Bacterial leaf blight, sheath blight paddy, damping off of chili wilt, banana, tomato, leaf rot groundnut, red rot of sugarcane	Rice, chickpea, vegetable	Sri Biotech Lab India Ltd. Hyderabad

et al. 2004). ISR conformity reflects physiological and biochemical reactions of the host plant, resulting in the synthesis and secretion of defense chemicals such as phenolic compounds, peroxidase, and phenylalanine ammonia lyase (PALase) enzymes (Van Loon et al. 1998).

Phenolic compounds function toward establishing antimicrobial activity, while peroxidase is a key enzyme in the biosynthesis of lignin and oxidation of hydroxylcinnamyl alcohols into free radical intermediates, which have been correlated with viral disease resistance (Saini et al. 1988; Bruce and West 1989). PALase, also known as phenylalanine ammonia lyase, is responsible for biosynthesis of various defense chemicals in phenylpropanoid metabolism and promotes plant functions that elicit strength and repair of the cell wall, antimicrobial activity, and signaling (Duijff et al. 1997). In addition, ISR-expressing plants have the capacity to convert aminocyclopropane-1-carboxylate (ACC), an essential precursor molecule to ethylene biosynthesis, which acts as a suppressant against phytopathogens during initial stages of pathogen attack (Niranjan Raj et al. 2005). Along with signaling SA compounds, *P. fluorescens* WCS417r ISR portfolio involves phytohormones jasmonic acid and ethylene as signals (De Meyer and Hofte 1997; Pieterse et al. 2003). Signaling compounds such as salicylic acid (SA) and ethylene (ET) play roles in regulating and inducing basal resistance. SA is a key regulator of systemic acquired resistance (SAR), while ET is initiated through rhizobacteria ISR (Niranjan Raj et al. 2005; Khan and Parmar 2013). Root colonization of *A. thaliana* by *P. fluorescens* WCS417r has shown to elicit ISR against *P. syringae* pv. tomato (Pst) (Knoester et al. 1999). SAR differs with respect to its capacity to be effective against pathogens through direct antibiosis by the PGPR, where the non-induced plant activities are controlled through SA-dependent defenses causing necrosis.

Similarly, ISR are effective against pathogens in non-induced plants and dependent on ET-producing compounds not causing any necrosis (Ton et al. 2002). Diminished ethylene production in roots/leaves and limited expression of the ethylene biosynthetic enzymes, ACC synthase and ACC oxidase, suggested the expression of ISR that requires complete submission of the signal transduction pathway (Knoester et al. 1999; Khan and Parmar 2013). *Pseudomonas fluorescens* WCS365 and *P. chlororaphis* PCL1391 have shown to suppress *Fusarium oxysporum* f. sp. *radicis-lycopersici* on tomato foot and root rot through the synthesis of antifungal metabolite phenazine-1-carboxamide (PCN). In the presence of *F. oxysporum* f. sp. *radicis-lycopersici*, tomato seedlings showed 70–90 % disease indication, while through treatment with WCS365 and PCL1391, disease suppression diminished to 0–15 % and 6–15 %, respectively (Dekkers et al. 2000; Jarvis 1988). ISR applications by *P. fluorescens* have shown to trigger pathogenic suppression, including suppression of *Arabidopsis thaliana* by *P. fluorescens* WCS365 (Gerrits and Weisbeek 1996). Cucumber seeds treated with *P. putida* 89B-27 reduced lesion diameters of angular leaf spot caused by invasive *P. syringae* pv. *lachrymans* (Liu et al. 1995). *Pseudomonas fluorescens* 97 suppressed *P. syringae* pv. *phaseolicola*, reducing the incidence of halo blight disease against beans. Similarly, seed treatment of rice by *P. fluorescens* Pf1 and

FP7 showed high levels of induction by ISR against sheath blight pathogen *R. solani* (Vidhyasekaran and Muthamilan 1999). *Pseudomonas* sp. WCS417r treatment of carnation plants was directed by ISR activity against *Fusarium* wilt caused by *Fusarium oxysporum* f. sp. *dianthi* (Van Peer et al. 1991). *Pseudomonas putida* BTP1 enhanced resistance through ISR against cucumber root rot caused by *Pythium aphanidermatum* and of bean against *Botrytis cinerea* (Ongena et al. 2002). In addition, *P. fluorescens* CHAO induced systemic resistance against necrosis virus (TNV) in tobacco; similarly, strain 89B-2 consistently reduced incidence of cucumber mosaic virus (CMV) and delayed the development of disease symptoms in cucumber and tomato (Raupach et al. 1996). Many applications of resistance have been identified through ISR, consistently providing optimal suppression, as well as secreting other molecular metabolites such as siderophores and phytohormones that assist in plant-microbe interaction and induced systemic resistance.

10.3.5 Bioinsecticidal Activity of *Pseudomonas*

Besides antiphytopathogenic activity against fungal disease, fluorescent pseudomonads show bioinsecticidal activity against agriculturally important insect pests such as aphids, beetles, and termites (Devi and Kothamasi 2009). Important strains of *Pseudomonas* showing insecticidal activity are listed in Table 10.3. *Pseudomonas entomophila* produces strong hemolytic and surfactant activity due to production of entolysin A, the cyclic lipopeptide of 14 amino acids and three C₁₀OH fatty acids synthesized by non-ribosomal peptide synthase (NRPS) system. *Pseudomonas entomophila* harbors five gene clusters associated with secondary metabolites such as HCN, lipopeptides, and polyketides (Vodovar et al. 2005). Entolysin A synthesis is encoded by *etlA* gene and involves two other genes *etlB* and *etlC*. Analogs of these genes are found in *P. putida* which synthesizes putisolvin with surfactant activity encoded by *psaB* and requires two other NRPS genes *psaA* and *psaC*. The surfactant activity and swarming mobility are correlated and are required for efficient colonization. Strains associated with entomopathogenicity harbor genes for a number of putative virulence factors (PVF) such as TccC-type toxin peculiar to entomopathogenic bacteria, *Photorhabdus luminescens* and *Xenorhabdus nematophila*, but absent in other *Pseudomonas* genomes (Hinchliffe et al. 2010). Proteases also contribute to virulence, and *P. entomophila* encodes three serine proteases (*pseent3027*, *preent3028*, *preent4433*) and an alkaline protease (*preent1150*) absent in *P. putida*. Some surface-associated factors allowing adhesion and effective colonization also contribute to pathogenesis (Table 10.3).

The genes coding for PVF have also been detected in *P. fluorescens*, *P. syringae*, and *Burkholderia cenocepacia*. Genomic sequencing of root colonizer, *P. fluorescens* strains CHAO and Pf5, now classified as *Pseudomonas protegens* (Ramette et al. 2011), has resulted in identification of a cluster of eight genes for synthesis of Fit, an insecticidal toxin similar to the product of *mcfl* gene of

Table 10.3 Important strains of *Pseudomonas* spp. showing insecticidal activity for biocontrol

Strain	Effector molecule/ metabolite	Mode of application	Target insect	References
<i>P. aeruginosa</i>				
CHA	T3SS and effectors (ExoS)	Pricking	<i>D. melanogaster</i>	Avet- Rochex et al. (2005)
PAO1	HCN, exotoxin A, GacA ExoS, pyoverdine, quorum sensing	Injection/ feeding	<i>D. melanogaster</i> <i>B. mori</i> <i>Pieris rapae</i>	Chieda et al. (2005, 2011) de Bentzmann et al. (2012) Vogt et al. (2011)
PA14	T3SS and effectors (ExoT and (ExoU), quorum sensing (RhIR)	Injection/ feeding	<i>G. mellonella</i> <i>D. melanogaster</i>	Miyata et al. (2003) Limmer et al. (2011)
<i>P. chlororaphis</i>				
PCC 1391 ST-1	Fit toxin ND	Feeding Injection	<i>S. litroratis</i> , <i>H. Virescens</i> <i>P. xylostella</i> <i>Bombyx mori</i>	Ruffner et al. (2013) Tao et al. (2011)
<i>P. entomophila</i>				
L48	Monalysin (pore- forming toxin) AprA (metalloprotease) GaCA, Pvf (signaling system) Alg R (regulator)	Feeding	<i>D. melanogaster</i> <i>G. mellonella</i>	Vodovar et al. (2005) Vullet-Gely et al. (2010) Fedhila et al. (2010)
<i>P. fluorescens</i>				
AH1, FP7, Pf 1	ND	Feeding	<i>Cnaphalocrocis medinalis</i>	Commare et al. (2002) Karthiba et al. (2010)
HS 870031	Viscosin (biosurfactant)	Contact (metabolite)	<i>M. persicae</i> , <i>A. gossypii</i> <i>Aulacorthum solani</i>	Hashimoto (2002)
KPM 018P	ND	Feeding	<i>Henosepilachna vigintioctopunctata</i>	Olcot et al. (2010)
<i>P. protegens</i>				
CHAO	Fit toxin (similar to Mcf toxin of <i>Photorhabdus</i>), GacA, HCN	Injection/ feeding/ contact	<i>G. mellonella</i> , <i>M. sexta</i> <i>S. littoralis</i> , <i>H. virescens</i> <i>P. xylostella</i>	Péchy-Tarr et al. (2008, 2013) Ruffner et al. (2013)

(continued)

Table 10.3 (continued)

Strain	Effector molecule/ metabolite	Mode of application	Target insect	References
Pf.5	Fit toxin, Gae A	Injection/ feeding	<i>G. mellonella</i> , <i>M. sexta</i> <i>D. melanogaster</i>	Péchy-Tarr et al. (2008) Olcot et al. (2010)
F6	Orfamide (surfactant)	Contact (metabolite)	<i>M. persica</i>	Jang et al. (2013)
<i>Pseudomonas</i> sp.				
EP-3 ICTB-745	Rhamnolipid Rhamnolipid, PCA (antibiotic)	Contact	<i>R. dominica</i>	Kamal et al. (2012)
<i>P. syringae</i>				
B728	Fli L (Flagellum and motility)	Feeding	<i>A. pisum</i>	Stavrinides et al. (2009)
<i>P. taiwanensis</i>				
TKU015	TccC-like toxin	Feeding	<i>D. melanogaster</i>	Liu et al. (2010)

Photorhabdus luminescens, pathogenic to insects (Péchy-Tarr et al. 2008). Gene fitD codes for the fit toxin (327 kDa) flanked by four genes *fitABCE* coding for type I secretion system and three genes *fit FGH* coding for regulatory proteins. Homologs of fit genes have also been detected in *P. chlororaphis* (Ruffner et al. 2013). They show high toxicity toward lepidopteran larvae, e.g., African cotton leafworm (*Spodoptera littoralis*), tobacco budworm (*Heliothis virescens*), and diamondback moth (*Plutella xylostella*) that feed on leaves. *Pseudomonas fluorescens* strains also kill *Drosophila melanogaster*, a common fruit fly (Olcot et al. 2010). A combination of two strains having insecticidal and antifungal properties resulted in reduction of incidence of rice leaf roller (*Cnaphalocrocis medinalis*) and phytopathogenic fungi, *Rhizoctonia solani*, on rice under greenhouse and field conditions (Commare et al. 2002; Karthiba et al. 2010). Biotechnological approaches have potential to enhance bioinsecticidal properties of fluorescent pseudomonads. Techniques of encapsulation of delta toxin of *Bacillus thuringiensis* or cloning of genes coding for crystal protein, Cry1Ac, have been used to enhance the efficacy of bioinsecticidal properties of *P. fluorescens* and *B. cepacia*. Such strains have been effectively used for control of lepidoptera and coleoptera insects (Joung & Côté 2002; Duan et al. 2004).

10.4 Plant Growth Regulators: Phytohormones

Phytohormones are described as plant growth-promoting hormones or chemical messengers active in regulating response to biotic and abiotic stresses through synergistic or antagonistic actions. This is referred to as signaling cross talk (Schmelz et al. 2003). Phytohormones affect the root systems directly by inducing cell elongation and cell division and differentiation or indirectly through ACC deaminase activity, promoting lateral root development (Patten and Glick 2002). Root elongation and subdivision enable nutrients and minerals to be sequestered from distant and localized regions, while ACC deaminase hydrolyzes plant ACC, thereby preventing inhibiting levels of phytohormone ethylene from acting antagonistically against the plant (Hong et al. 1991; Patten and Glick 2002). *Pseudomonas putida* UW4, a novel bacterium affixed with ACC deaminase abilities, have shown versatile characteristics, having plant growth under harsh environmental conditions such as flooding, drought, the presence of heavy metals, and the presence of phytopathogens (Godinho and Bhosle 2013).

In addition to ACC activity, the ability to synthesize phytohormones has been widely recognized as an attribute, where 80 % of PGPR are able to produce auxins (Costacurta and Vanderleyden 1995). Auxins are defined for their characteristic as a plant hormone containing indole-3-acetic acid (IAA), which through synthesis acts as a signal molecule in the regulation of plant development, stimulating organogenesis, rapid cell growth, division, and differentiation (Cleland 1990). Tryptophan (L-Trp) is an amino acid readily secreted in root exudates, generally recognized as the precursor molecule for promoting IAA synthesis (Costacurta and Vanderleyden 1995). *Pseudomonas syringae* (Hutcheson and Kosuge 1985), *P. fluorescens*, and *P. putida* (Khakipour et al. 2008) are the most important and efficient type of auxin synthesizers, using L-tryptophan as the precursor molecule recognized for plant growth promotion. *Pseudomonas fluorescens* isolates were tested for their ability to produce IAA in pure culture, in the presence and absence of L-tryptophan. Results suggested an increase in the production of IAA directly correlated to increases in tryptophan (Karnwal 2009).

Similarly, production of IAA in *Pyrus* (pears) and *Malus* (apple) by the fluorescent pseudomonads were quantified, showing auxin concentrations in normal sites of respective rhizospheres ranging from 7-30 µg/ml. Auxin extracted from *Pseudomonas fluorescens* cultures provided exceptional results using the *Avena* coleoptiles straight growth test and found increased length of coleoptiles ranging from 0.2 to 0.25 cm (Kapoor et al. 2012). Ali et al. (2009) studied the impact of auxin production on endogenous IAA content and growth of *Triticum aestivum* L.; *Pseudomonas* sp. strains As-17 and AvH-4 increased endogenous IAA by 208 and 187 %, respectively, with the highest increase in the number of roots over water-treated controls reported as 83 % from 25 % for As-17 and maximum increase in root length and fresh weight attributed to strain AvH-4, 40 % over control.

Inoculation of *Brassica campestris* (canola seeds) with *Pseudomonas putida* strain GR12-2 traditionally produces low levels of IAA, resulting in a two- to

fourfold increase in the length of seedling roots. However, testing the impact of IAA overproduction, inhibition of root growth of seedlings accounted for 33 % (Xie et al. 1996; Glick et al. 1986). In addition, higher concentrations of tryptophan correlate to higher levels of IAA produced, thus exerting an inhibitory effect on plant growth parameters (Ahmad et al. 2005). However, coinoculation with most IAA-producing strains of *Pseudomonas* spp. with *Rhizobium* has shown to benefit plant growth promotion by enhancing the production of flavonoids or phytoalexins, which have been associated in additional factors that promote nodule formation (Parmar and Dufresne 2011). Coinoculation with *Mesorhizobium* sp. *Cicer* strain Ca181 resulted in an increased number of nodules and nodule fresh weight. Coinoculation with *Pseudomonas* sp. isolates MPS79 and CPS10, which overproduced levels of IAA, causing a stunting effect on shoot under culture conditions, resulted in a significant gain in shoot dry weight 100 days from the initial inoculums (Malik and Sindhu 2011).

10.5 Engineering Transgenic Plants with Replicase- and RNA-Mediated Resistance

Replicase-mediated resistance is a gene-specific method that can confer near immunity to infectious genes through its capacity to encode complete or partial replicase proteins. This method is highly effective limiting the virus strain from which the gene sequence was obtained (Beachy 1997). Replicase-mediated resistance (Rep-MR) to TMV was first described in transgenic plants containing a sequence encoding a 54 kDa fragment of replicase (Golemboski et al. 1990). It was suggested that the protein produced by the transgene interferes with the virus replicase by binding to host factors or virus proteins that regulate replication and gene expression (Beachy 1997). Donson et al. (1993) demonstrated this theory by inserting a bacterial transposable element into a TMV replicase transgene during propagation in bacterial hosts. The site of the transposon insertion and properties of the replicase fusion protein product enabled resistance to be administered against TMV and tobamoviruses. Zaitlin et al. (1994) described the mechanism of Rep-MR using a truncated mutant replicase derived from cucumber mosaic virus (CMV) subgroup I and supplementing it into tobacco plants. The mutant replicase conferred high levels of resistance to all subgroup I CMV strains and Rep-MR against CMV which also confirmed sustained inhibition of virus accumulation and systemic infection (Hellwald and Palukaitis 1995).

Geminiviral Rep proteins have also been widely used to illustrate engineered pathogen resistance. The Rep gene of African cassava mosaic virus (ACMV) inhibited virus replication in protoplasts and induced virus resistance in plants (Hong and Stanley 1995). Similarly, a truncated tomato yellow leaf curl Sardinia virus (TYLCSV) mutant Rep protein, lacking a conserved NTP-binding terminal domain, strongly inhibited virus replication in protoplasts, suppressing the expression of viral Rep protein and forming dysfunctional complexes with the viral Rep protein. As a result, a high level of resistance was confirmed through sequence-

specific targeting of the TYLCSV Rep protein (Noris et al. 1996). Further analysis into Rep-MR by Audy et al. (1994) using potato virus Y (PVY) and Brederode et al. (1995) using alfalfa mosaic virus (AIMV) revealed that resistance was mediated through defects in the primary protein structure encoded by the transgene. This revelation was confirmed by using transgenes encoding the wild-type version of replicase protein which was unable to inhibit resistance; however, when tested against mutant replicase protein, a high degree of resistance was witnessed (Baulcombe 1996). These mechanisms of Rep-MR through protein or nucleic acid targeting enable biotechnologists to selectively target the transgene replicase protein, engineer it to suspend viral transcription upon contact, and effectively inhibit incidence of infection.

In light of such findings, another avenue biotechnologist can offer to confer a high degree of resistance stems toward the direct inhibition of the viral infection cycle by the transgene or RNA transcript itself; this mechanism is known as RNA-mediated resistance. The RNA-mediated resistance method orients itself around posttranscriptional gene silencing, also known as RNA interference/silencing (RNAi) where a sequence-specific RNA strand is broken down and rendered inactive (Prins et al. 2008). The mechanism, as demonstrated by Lindbo et al. (1993), conferred resistance using transgenic tobacco plants against potato Y potyvirus by sequence-specific RNA degradation in the cytoplasm. RNA degradation will target RNAs with sequence identity complimentary to the transgene RNA (Prins et al. 2008). Furthermore, through run-on labeling experiments using the potyvirus-resistant tobacco plants, the rate of transcription in the nuclei of resistant plant cells is relatively high, compared against the nuclei of susceptible transgenic plants (Lindbo and Dougherty (1992). Levels of transgenic mRNA in resistant plant cells exist as relatively low, at times even lower than those in the susceptible transgenic plants. This suggests that activity in the cytoplasm of the resistant plant cells mark the selective and rapid degradation of transgenic mRNA and homologous RNA of invading viruses (Smith et al. 1994; Mueller et al. 1995).

Furthermore, the basis of sequence-specific recognition relies on the generation of small interfering RNA (siRNA) molecules derived from the transgene, which could be found on wild-type plants infected with viruses and viroids (Hamilton and Baulcombe 1996). The presence of siRNA molecules generated in transgenic plants and on the wild-type plant suggests that the RNA-induced silencing complex (RISC) mediates antiviral recognition prior to infection. As such, viral RNA molecules are rapidly and effectively targeted and degraded, even before the virus-encoded RNA silencing suppressor proteins are produced (Baulcombe 1996; Prins et al. 2008). Sequence-specific resistance can be obtained in all transgenic plants that confer at least 90 % nucleotide sequence homology between transgene and corresponding viral gene (Zaitlin et al. 1994). It was suggested that this mechanism of engineered PDR cannot simply be overcome by pathotypes with single or few point mutations in the genome, as 90 % of the nucleotide sequence remains intact; thus, corresponding similarity is required for RNA-mediated resistance (De Haan 1998).

10.5.1 Current Development in Transgenic Crops

The revelation of optimizing growth, fertility, and self-regulated biocontrol through production of transgenic crops has generated much interest in the agricultural field and continues to grow. Through recognition from the FDA, the existence and marketing of 40 transgenic crops currently implemented in crop cultivars around the world (Whitman 2000; Mellon and Rissler 2003). The advantages of transgenesis include increased biotic resistance to pests; increased herbicide tolerance; enhanced disease resistance; affective control against abiotic stresses such as drought, cold, and salinity; and increased nutrition synthesis and production (Whitman 2000). Much of the work, however, has been marketed toward eliminating the use of chemical pesticides through engineering of insecticidal proteins and developing environmental tolerance.

Helicoverpa armigera (pod borer) and a homopteran group of sucking insects, *Aphis craccivora*, represent two of the most potent pests causing damage to pulse crops globally (Das 2005). *Helicoverpa armigera* in chickpea alone has resulted in yield losses up to 40 % in many Indian farms with a worldwide loss of profit estimated over US \$330 million annually (Reed et al. 1987; ICRISAT 1992). To mitigate this loss, the development of transgenic plants expressing insecticidal proteins was proposed. Using insecticidal Cry proteins derived from *Bacillus thuringiensis* (Bt), resistance was administered directly through the ingestion of the Bt protein and indirectly by infected prey-mediated interactions (Cowgill and Lateef 1996). These Cry proteins are classified as δ -endotoxins that bind to the midgut epithelial cells, inducing osmotic lysis in the invading pest, causing reduced activity and eventual demise (Herrera-Estrella et al. 2005).

Currently, over 14,000 chickpea germplasm accessions and breeding lines have been screened for resistance against *H. armigera* at ICRISAT (Lateef and Sachan 1990). High-expressing Bt lines expressing the *cry2Aa* gene isolated by Sarmah (2006) have shown to confer near complete protection, reporting a 98 % overall mortality of *H. armigera* larvae. Similarly, these Bt lines in transgenic plants expressing δ -endotoxins are employed toward promoting active transgenesis in tobacco, tomato, cotton, potato, maize, canola, soybean, rice, sugarcane, and chickpea (Herrera-Estrella et al. 2005). Continuous work to broaden the scope of transgenesis and insecticidal resistance is being marketed for commercial application. Chickpea, tobacco, and through nitrogen fixation and phosphorus solubilization, among other factors, tomato plants expressing different *cry* genes have shown positive resistance in trial runs. To be implemented commercially, more work to justify consistency and management needs to be done (McPhee et al. 2007). Currently, the only Bt-transgenic crops available for commercial application against *H. armigera* are cotton, maize, and potato (Herrera-Estrella et al. 2005; James 2002; Quaim and Zilberman 2003). Through continuous support and research, marketability, and application an improvement in fertility, resistance and yield can be achieved. Eliminating pesticide and chemical sprays through means of transgenic plants, self-regulating resistance, and control encourages the viability of such technologies, without sacrificing quality and safety of the product.

10.6 Conclusions

Pseudomonas spp. have been recognized as one of the most prominent rhizosphere colonizing species capable of establishing colonial dominance within the rhizosphere either native or foreign to their ecological niche. Through various mechanisms of growth promotion, biostimulation and augmentation of the rhizosphere, and biological induction of invasive phytopathogens, *Pseudomonas* spp. have revolutionized the agricultural business. Phosphate solubilization and production of secondary metabolites promote aggressive growth and colonization as seen with increases in siderophore activity, readily secreting hormones that direct root and shoot growth through cellular differentiation and division. This mediates an increased effectiveness to uptake exudates, chelate iron compounds, and secrete biologically induced compounds alongside antimetabolites, such as antibiotics, HCN, and siderophores. The antifungal activity of *P. fluorescens* is correlated to the production of two important secondary metabolites, 2,4 diacetylphloroglucinol (Phl) and phenazine besides a number of other factors regulated by GacS/GacA complex and environmental responses. In addition, plant-microbe feedback through nitrogen fixation using *P. stutzeri* is another mediated factor, a field relatively absent or inadequate, that has shown tremendous gains, ultimately promoting plant growth. Biological growth-inducing factors and physiological RNA-mediated resistance show increasing trends where biotechnologists harbor beneficial genes and gene segments that initiate specific response beneficial to the fertility of the plant and competency of the PGPR within the rhizosphere. This leads to the development of a genetic profile of the soil, where competent and favorable traits are recognized to influence growth and colonization, while tactically suppressing and killing phytopathogens. The production of transgenic crops have also been rationalized, with trials optimizing growth in tobacco, tomato, cotton, potato, maize, canola, soybean, rice, sugarcane, and chickpea already undertaken showing positive results. Continuous improvement, optimization, and success using biologically competent *Pseudomonas* spp. have paved the path to challenge the paradigm of traditional practices, with the results ultimately determining the viability of such technologies, ensuring quality is not sacrificed and the system remains viable.

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Rachhpal S. Kahlon

Contents

11.1	Introduction	470
11.2	Taxonomy of <i>Pseudomonas Syringae</i>	471
11.3	Plant Pathogenesis: An Overview	479
11.3.1	Entry of <i>Pseudomonas syringae</i>	480
11.3.2	Plant Immune Response Against <i>P. syringae</i>	481
11.3.3	Overcoming Resistance of Host Plant and Pathogenesis	482
11.4	Virulence Factor and Molecular Mechanism	482
11.5	Type III Secretion System and Effectors (TTSS and TTSE)	483
11.5.1	Structure of TTSS	485
11.5.2	Regulation of TTSS	486
11.6	Toxins and TTSS Independent Virulence Factors	488
11.6.1	Coronatine	488
11.6.2	Lipodepsipeptide Toxins: Syringomycins and Syringopeptins	492
11.6.3	Antimetabolite Toxins	498
11.6.4	Phytohormones and Other Mechanisms	502
11.6.5	Exopolysaccharide	503
11.7	Genomics of <i>Pseudomonas syringae</i>	505
11.7.1	Plasmids of <i>P. syringae</i> and Pathogenicity	508
11.7.2	Biotechnology for Novel Bioactive Compounds and Transgenic Plants	510
	References	511

Abstract

Pseudomonas syringae is pathogenic to a wide range of plants including field crops, vegetables, fruit trees and garden plants causing huge economic losses in agriculture. The strains of *P. syringae* isolated from a particular plant type are highly specific and are subdivided into nearly 50 pathovars depending upon the host plant. These have been grouped into five phylogenetic clades on the basis of

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multilocus sequence type (MLST) analysis, representing nine genomospecies based on DNA hybridisation studies. Plant pathogenesis is a complex process with plants resisting infection by an innate immune system comprising of pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). On the other hand, the pathogen suppresses the innate immunity by producing a number of effector molecules (proteins) which are lodged into the cytoplasm of the host cell by a specialised structure, the injectisome formed by TTSS. The TTSS effectors produced by *P. syringae* are enzymes such as protease, hydrolase, phosphatase, kinase, etc. which biochemically modified the host molecules by disrupting their function or eliminating them. Effector molecules have emerged central to plant microbe interaction. Besides *P. syringae* also produces a number of antimetabolite toxins, viz. coronatine, syringomycin, syringopeptin, tabtoxin, etc. Some of the host cell proteins, helpers, contribute to effector maturation in the cytoplasm and act as factors to form biochemically active complexes. Effectors are also being used as molecular probes to study plant biology and plant microbe interactions. Engineered beneficial synthetic effectors can be useful in biotechnology and systems biology to bring about specific changes in genome.

11.1 Introduction

All plants in nature grow in the tripartite micro-ecological environment comprising of soil, microorganisms and nutrients as solutes in water and gases which maintain a desirable balance of physicochemical properties and a physiological state. Thus microorganisms, being always there in the plant growth environment, interact with plants in one of the three different ways:

1. Commensals, in which case the colonising bacteria draw their nutrients from the plants without causing any damage or deleterious effect to the host plant.
2. Mutualists, which exert a positive effect on plant health and development. The closest form of mutualism is a symbiotic relationship, where both the host plant and the microbe benefit from their relationship.
3. Pathogens, which cause a visible damage to the host plant and parasitise the host plant for its requirement of nutrients, etc.

These interactions can't be considered as absolute but display a continuum as symbiotic and pathogenic microorganisms are present in the same ecosystems as commensals till they reach and enter the closer association to establish symbiotic or pathogenic relationship and some organisms may even switch between different states (Hirsch 2004; Newton et al. 2010), i.e. a commensal may turn to a parasite. Thus the process and nature of interaction involves the expression and regulation of the synthesis of specific gene products to establish the obvious relationship.

Among the large number of microorganisms that inhabit the micro- ecological environment of the plant, *Pseudomonas* is the most versatile and metabolically

diverse. Now, over 200 species are classified under the genus *Pseudomonas*, many of which are important from the point of view of the environment due to their capacity to degrade a variety of toxic and hazardous compounds in the environment, some are important as colonisers of the plant surface and rhizosphere and promote plant growth either directly or by suppression and biocontrol of plant diseases, some are pathogenic to humans, animals and plants and a few have even shown potential for commercial exploitation for production of industrially important chemicals, enzymes and processes, etc. Only one species, *Pseudomonas syringae*, is known to be pathogenic to a variety of plants and the strains are highly specific for the host plant and are designated as pathovars within the *P. syringae* complex species depending on the plant type to which one is pathogenic and cause disease. Currently about 50 pathovars isolated from 180 different plant types are recognised (Gardan et al. 1999). *Pseudomonas syringae* mainly infects aerial parts such as leaves and fruits and infection remains localised as infection lesions are self-contained within few millimetres of the initial site of infection. It does not spread to other parts of the plants. The initial stage is epiphytic, in which the *P. syringae* resides on the surface of a leaf or other aerial parts of the healthy plant and then in the endophytic phase in the apoplastic space after entry into the plant through stomatal openings or injury on the surface of the plant surface. The cells multiply vigorously in the living host cell till they reach the peak levels. The host cell may die at this stage and show extensive necrosis and lesions of the disease. As such they are referred as hemi-biotrophic to distinguish from strictly biotrophic pathogens which obtain nutrients from living host cells without causing death of the host cell and strict necrotrophic pathogens which kill the host cells during early stages of infection for drawing nutrients.

11.2 Taxonomy of *Pseudomonas Syringae*

Members of the genus *Pseudomonas* show a high degree of ecological and cultural variability and nutritional diversity. The genus, in itself, was vaguely defined as gram-negative rods, aerobic with chemoorganotrophic metabolism and motile with one or more polar flagella (Migula 1894). A large number of gram-negative bacteria fall in this category and were assigned to this genus. However, with the development of molecular techniques and analysis of 16S rDNA (Palleroni et al. 1973), taxonomy of *Pseudomonas* has undergone extensive changes. All known pseudomonads on the basis of comparative 16S-rDNA hybridisation analysis were differentiated into five different groups under class Proteobacteria, and the genus *Pseudomonas* sensu stricto represents the rRNA group I, subclass Gammaproteobacteria with *Pseudomonas aeruginosa* as type species. All other strains which belonged to rRNA groups II–V on the basis of hybridisation studies were referred as *Pseudomonas* sensu lato and classified under other subclasses of Proteobacteria (for detail, refer to Chap. 1).

Kerstens et al. (1996) assigned the members of the genus *Pseudomonas* to different subclasses, families and genera under Proteobacteria. Most of the

pseudomonads are saprophytic and widely distributed in soil, water and air; one species, *P. aeruginosa*, is an opportunistic pathogen of humans and animals as it causes cystic fibrosis and wound infection and is notorious as nosocomial infections because of its multiple-drug resistance. While *Pseudomonas syringae*, a complex species, and related species are pathogenic to field crops, vegetables and fruit plants (*P. syringae*, *P. savastanoi*, *P. marginalis* and *P. cichorii*) or mushrooms (*P. agarici* and *P. tolaasii*) and are responsible for agricultural losses by causing speck, spot and blight diseases.

Until the 1960s most of the plant-pathogenic pseudomonads were identified on the basis of their ability to infect a particular host plant, e.g. *Pseudomonas mori* was infective to *Morus* spp. and the strains were host specific. *Pseudomonas syringae* was first isolated from lilac. Thus pathogenicity gained precedence over other characteristics for their identifications (Burkholder and Starr 1948). Other characters considered for pathogenic bacteria were morphological, biochemical and nutritional tests and colony characteristics on different media. Lelliott et al. (1966) showed that five tests were important to differentiate fluorescent plant-pathogenic *Pseudomonas*. These five tests, production of levan, oxidase activity, capacity to rot potato, production of arginine dihydrolase and hypersensitivity reaction to tobacco (LOPAT), differentiated the plant-pathogenic species into five different groups. Species showing negative tests for oxidase activity, capacity to rot potato, production of arginine dihydrolase and positive hypersensitivity reaction to tobacco were classed as LOPAT group I pathogens. However, extensive studies by Sands et al. (1970) and DNA–DNA hybridisation studies (Palleroni et al. 1972) indicated a genomic diversity within LOPAT group I. Fluorescent phytopathogenic pseudomonads cluster within species of the genus *Pseudomonas* sensu stricto with 16S-rRNA similarity group I. The eighth edition of Bergey's Manual of Determinative Bacteriology included most of fluorescent phytopathogenic pseudomonads as *Pseudomonas* nomenspecies (Doudoroff and Palleroni 1974). In the first edition of Bergey's Manual of Systematic Bacteriology (Palleroni 1984), 41 nomenspecies were included as pathovars of *P. syringae* where reference strains were referred as pathotype strains (Dye et al. 1980). Therefore, *P. syringae*, *P. amygdali*, *P. meliae*, *P. coronafaciens*, *P. ficuserectae* and *P. viridiflava* were the only fluorescent, oxidase-negative phytopathogens included in the approved list. These pathovars could not be identified by routine biochemical tests.

Gardan et al. (1999) undertook analysis of DNA relatedness of 48 pathovars of *P. syringae* and eight related species. Of these 51 belonged to six DNA hybridisation groups referred as genomospecies. DNA hybridisation group I included ten strains of different pathovars of *P. syringae* showing 71–100% relatedness to *P. syringae* CFBP1392^T. Genomospecies 1 corresponded to *P. syringae* sensu stricto. Thus, *P. syringae* and pathovars *aptata*, *lapsa*, *papulans*, *pisi*, *atrofaciens*, *aceris*, *dysoxyli* and *japonica* belong to *P. syringae* sensu stricto, also referred as DNA–DNA hybridisation group syringae. Group II included 20 strains: 16 pathovars of *P. syringae* and type strains of related species, *P. savastanoi* (CFBP-1670^T), *P. ficuserectae* (CFBP3224^T), *P. meliae* (CFBP3225^T) and *P. amygdali* (CFBP3340^T). They showed 72–100% DNA relatedness to type

strain *P. savastanoi*, CFBP-1670^T. Three strains of *P. savastanoi* used in this study were 79–93 % related to strain CFBP-1670^T.

The strains of species *P. savastanoi*, *P. ficuserectae*, *P. meliae* and *P. amygdali* included in genomospecies 2 are considered synonymous and could be named *P. amygdali*.

DNA hybridisation group III included 14 strains of *P. syringae* that showed 71–100 % similarity to the type strain *P. syringae* pv. *tomato* CFBP2212. Three strains of pathovar tomato showed 92–98 % relatedness to CFBP-2212. *Pseudomonas syringae* pv. *tomato* strain CFBP2212 has been suggested as a type strain of genomospecies 3.

DNA hybridisation group IV comprised of *P. syringae* pv. *coronafaciens* (*P. coronafaciens*) and seven other strains showing 78–100 % relatedness to *P. syringae* pv. *porri* CFBP1908. Three strains of *P. syringae* pv. *porri* used as control indicated 90–98 % relatedness to CFBP-1908. *Pseudomonas coronafaciens* is presumed to represent genomospecies 4.

Group V of DNA hybridisation comprised of *P. syringae* pv. *tremae* CFBP3229 which shows less than 20 % relatedness to other strains tested. This pathovar was therefore given the species status as *Pseudomonas tremae*. DNA hybridisation group VI included three strains, *P. viridiflava*, *P. syringae* pv. *ribicola* and *P. syringae* pv. *primulae* that demonstrated 71–100 % relatedness to *P. viridiflava* strain CFBP-2107^T. Thus genomospecies 6 represents *P. viridiflava*.

Pseudomonas syringae pvs. *tagetis*, *helianthi*, *theae*, *avellanae* and *cannabina* showing nearly 50% or less homology with six strains tested (Table 11.1) were subjected to hybridisation with ³H-labelled DNA from these pathovars. The 15 strains tested were grouped under genomospecies 7, 8 and 9. Two strains of *P. syringae* pv. *tagetis* and three strains of *P. syringae* pv. *helianthi* were grouped in genomospecies 7 with strain CFBP1694 as the type strain of *P. syringae* pv. *tagetis*. Three strains of *P. syringae* pv. *theae* (2353, 11012, 11013) and four strains of *P. avellanae* (11144, 11067, 11066, 2637) are included in DNA hybridisation group VIII. *Pseudomonas avellanae* strains showed 73–100 % relatedness with CFBP-11144 (*P. avellanae*). However, 84–100 % relatedness was observed among strains of *P. syringae* pv. *theae*. But DNA hybridisation with *P. avellanae* strains showed 66–78 % relatedness. Genomospecies 8 is represented by *P. avellanae*. DNA hybridisation group IX comprises of three strains, 2341, 1619 and 1631, of *P. syringae* pv. *cannabina*. The genomospecies has been named as *P. cannabina*.

The nine genomospecies based on DNA hybridisation cannot be differentiated on the basis of phenotypic tests as no reliable data is available to correlate substrate utilisation, biochemical tests and cultural characteristics with plant pathogenesis (Gardan et al. 1999).

Phylogenetic analysis of 56 strains of *P. syringae* belonging to 19 pathovars using two core genes, *gyrB* and *rpoD*, and two pathology-related genes, *hrpL* and *hrpS* (Sawada et al. 1999), were undertaken. Data indicated three distinct monophyletic groups.

Table 11.1 Different pathovars of *Pseudomonas syringae*, their host plant, symptoms of the diseases and relationship between the phylogroup and the genomospecies

NCPPB	Pathovar	Host	Disease/symptom	Genomospecies
Phylogroup I				
3739	<i>actinidiae</i>	<i>Actinidia chinensis</i>	Bacterial canker of kiwi fruit	8
1817	<i>antirrhini</i>	<i>Antirrhinum majus</i>	Leaf spot	3
1626	<i>apii</i>	<i>Apium graveolens</i> var. <i>dulce</i>	Leaf spot	3
2724	<i>berberidis</i>	<i>Berberis</i> sp.	Leaf spot	3
1879	<i>delphini</i>	<i>Delphinium</i> sp.	Leaf spot	3
2039	<i>maculicola</i>	<i>Brassica oleracea</i> var. <i>botrytis</i>	Bacterial spotting	3
2995	<i>morsprunorum</i>	<i>Prunus domestica</i>	Leaf spot and stem canker	8
1387	<i>passiflorae</i>	<i>Passiflora edulis</i>	Necrotic spots	3
2761	<i>persicae</i>	<i>Prunus persica</i>	Leaf spot, canker, dieback	3
CFBP5524	<i>spinaceae</i>	<i>Spinacia oleracea</i>	Leaf spot	3
2598	<i>theae</i>	<i>Thea sinensis</i>	Shoot blight, stem blight	8
1106	<i>tomato</i>	<i>Solanum lycopersicum</i>	Bacterial speck and leaf spot	3
1921	<i>viburni</i>	<i>Viburnum</i> sp.		3
4290	<i>avii</i>	<i>Prunus avium</i>		3
Phylogroup II				
958	<i>aceris</i>	<i>Acer</i> sp.	Leaf spot	1
871	<i>aptata</i>	<i>Beta vulgaris</i>	Leaf spot, foliar blight	1
2612	<i>atrofaciens</i>	<i>Triticum aestivum</i>	Leaf spot, basal glume rot	1
225	<i>dysoxyl</i>	<i>Dysoxylum spectabile</i>	Leaf spot, shoot hole	1
2096	<i>lapsa</i>	Corn hybrid seed	Stalk rot	1
2848	<i>papulans</i>	<i>Malus sylvestris</i>	Blister spot, blister canker	1
2585	<i>pisi</i>	<i>Pisum sativum</i>	Bacterial blight	1
281	<i>syringae</i>	<i>Syringa vulgaris</i> (wild host range)	Leaf spot, cankers, dieback	1
3093	<i>japonica</i>	<i>Hordeum vulgare</i>		1
Phylogroup III				
3681	<i>aesculi</i>	<i>Aesculus hippocastanum</i>	Leaf stop	2
ICMP 13650	<i>broussonetiae</i>	<i>Broussonetia kazinoki</i> x <i>B. papyrifera</i>	Bacterial blight	2
ICMP 9419	<i>castaneae</i>	<i>Castanea sativa</i>	Bacterial canker	2

(continued)

Table 11.1 (continued)

NCPBPB	Pathovar	Host	Disease/symptom	Genomospecies
ICMP 17524	<i>cerasicola</i>	<i>Prunus yecloensis</i>	Galls	2
2355	<i>ciccaronei</i>	<i>Cerantonis siliqua</i>	Leaf spot	2
ICMP 11894	<i>cunninghamiae</i>	<i>Cunninghamia lanceolata</i>		2
3617	<i>daphniphylli</i>	<i>Daphniphyllum teijsmanni</i>	Galls	2
3464	<i>dendropanacis</i>	<i>Dendropanax trifidus</i>		2
2331	<i>eriobotryae</i>	<i>Eriobotrya japonica</i>	Bud blight, twig canker	2
3682	<i>hibisci</i>	<i>Hibiscus rosa sinensis</i>	Leaf spot	2
2356	<i>mellea</i>	<i>Nicotiana tabacum</i>	Wisconsin tobacco disease	2
1034	<i>mori</i>	<i>Morus alba</i>	Leaf spot, shoot blight	2
3143	<i>myricae</i>	<i>Myrica rubra</i>	Galls	2
52	<i>phaseolicola</i>	<i>Phaseolus vulgaris</i>	Halo blight	2
3688	<i>photinae</i>	<i>Photinia glabra</i>	Leaf spot and blight	2
3618	<i>rhapiolepidis</i>	<i>Rhapiolepis umbellata</i>		2
1016	<i>sesami</i>	<i>Sesamum indicum</i>	Leaf spot	2
1427	<i>tabaci</i>	<i>Nicotiana tabacum</i>	Wildfire angular leaf spot	2
632	<i>ulmi</i>	<i>Ulmus</i> sp.; <i>Arabidopsis</i>	Leaf and shoot blight	3
<i>P. savastanoi</i> pathovars				
639	<i>savastanoi</i>	<i>Olea europaea</i>	Galls	2
52	<i>phaseolicola</i>	<i>Phaseolus vulgaris</i>	Halo blight	2
3278	<i>nerii</i>	<i>Nerium oleander</i>	Galls	2
4050	<i>retacarpa</i>	<i>Retama sphaerocarpa</i>	Galls	2
ICMP 7711	<i>fraxini</i>	<i>Fraxinus excelsior</i>	Galls	2
2411	<i>glycinea</i>	<i>Glycine max</i>	Bacterial blight	2
Phylogroup IV				
2397	<i>atropurpurea</i>	<i>Lolium multiflorum</i>	Halo blight	4
600	<i>coronafaciens</i>	<i>Avena sativa</i>	Halo blight	4
588	<i>garcae</i>	<i>Coffea arabica</i>	Halo blight	4
3683	<i>oryzae</i>	<i>Oryza sativa</i>	Halo blight	4
3690	<i>zizaniae</i>	<i>Zizania aquatica</i> — wild rice		4
3364	<i>porri</i>	<i>Allium ampeloprasum</i>	Bacterial blight	4

(continued)

Table 11.1 (continued)

NCPPB	Pathovar	Host	Disease/symptom	Genomospecies
Phylogroup V				
1437	<i>alisalensis</i>	<i>Brassica rapa</i>	Bacterial blight	9
3781	<i>coriandricola</i>	<i>Coriandrum sativum</i> var. <i>micocarpur</i>	Umbel blight, seed decay	9
3257	<i>philadelphi</i>	<i>Philadelphus coronarius</i>	Leaf spot	9
Phylogroup VI				
2640	<i>helianthi</i>	<i>Helianthus annuus</i>	Leaf spot	7
2488	<i>tagetis</i>	<i>Tagetes erecta</i>	Shoot blight, stem blight	7
Grouped with <i>P. viridiflava</i>				
133	<i>primulae</i>	<i>Primula</i> sp.	Leaf spot	6
963	<i>ribicola</i>	<i>Ribes aureum</i>	Leaf spot, defoliation	6

Group I comprised of pathovars: pv. *tomato*, pv. *morsprunorum*, pv. *syringae*, pv. *actinidiae* and pv. *theae*.

Group II comprised of pathovars: pv. *aceris*, pv. *aptata*, pv. *japonica*, pv. *syringae* and pv. *pisi*.

Group III comprised of pathovars: pv. *myricae*, pv. *erobotryae*, pv. *morsprunorum*, pv. *tabaci*, pv. *lachrymans*, pv. *castaneae*, pv. *phaseolicola*, pv. *glycinea*, pv. *mori* and pv. *broussonetiae*.

Strains of three pathovars, pv. *lachrymans*, pv. *morsprunorum* and pv. *syringae*, were distributed over two groups. Pathotype strains of pv. *lachrymans* and *morsprunorum* were included in group I, but all other strains were put in group III. For *P. syringae* only Japanese citrus strains belonged to group I while all others were included in group II. Group I corresponds to genomospecies 3 and 8 and group II corresponds to genomospecies 1 as defined by Gardan et al. (1999) (Fig. 11.1).

Mulet et al. (2010) undertook analysis of gene sequences of four housekeeping genes, viz. *16S rRNA*, *gyrB*, *rpoB* and *rpoD*. These genes code for the 16S rRNA sequence, *gyrB* codes for the β -subunit of DNA unwinding enzyme gyrase, *rpoB* codes for the β -subunit of RNA polymerase, and *rpoD* encodes the sigma-70 subunit of RNA polymerase. The phylogenetic tree (unrooted) of 107 strains of *Pseudomonas* showed that the *Pseudomonas syringae* group comprises of 12 species as deduced from the sequence analysis of four concatenated genes (*16S rRNA*, *gyrB*, *rpoB* and *rpoD*). The genomospecies based on DNA–DNA hybridisation studies were not supported by the ribotyping or biotype analysis of carbon source utilisation. Most of the pathovars fall under genomospecies 1–4 and only a small number of pathogens is represented by genomospecies 5–9. A comprehensive table has been provided by Young (2010). For the identification of *P. syringae* pathovars,

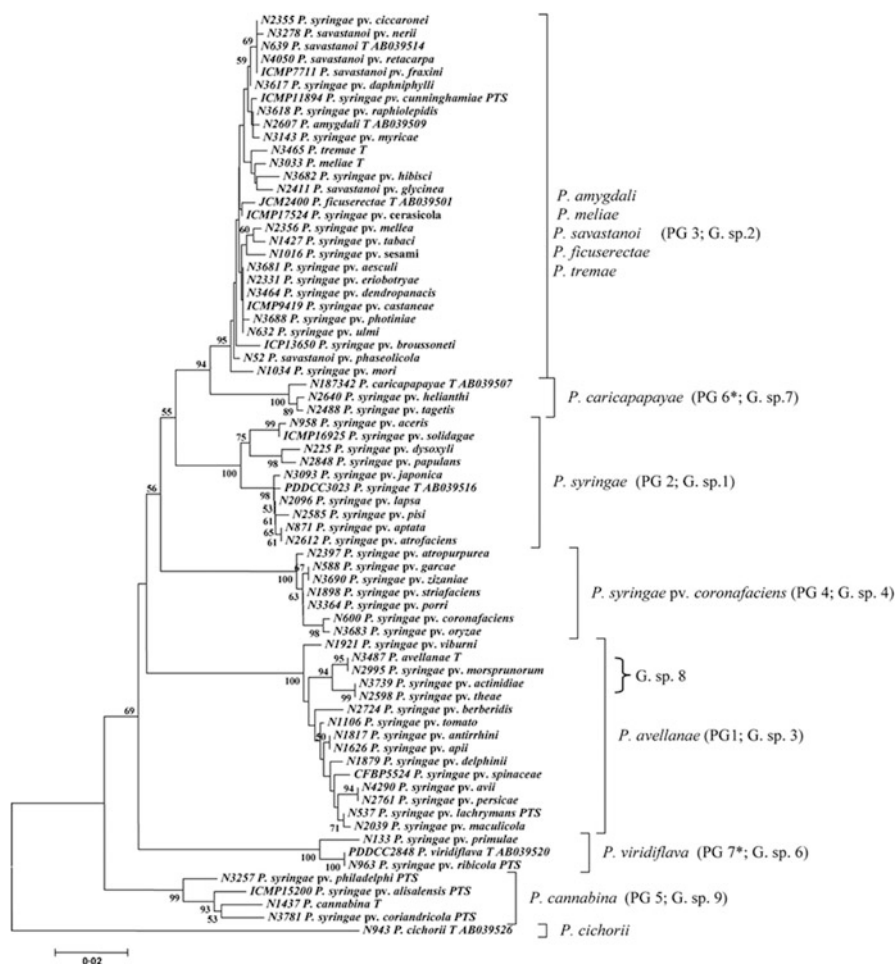


Fig. 11.1 Phylogenetic relationship between different pathovars of *P. syringae* and related species (With permission, Parkinson et al. 2011)

the use of determinative tests was found to have limited value for differentiation of pathovars which were based on their host species (Young and Triggs 1994; Young 2000, 2008; Palleroni 2008).

Palacio-Bielsa et al. (2009) have described 30 PCR primers for 19 members of *P. syringae* complex which included the three species *P. avellanae*, *P. cannabina* and *P. fuscovaginae* and 16 pathovars, viz. *actinidiae*, *alisalense*, *atropurpurea*, *coryli*, *glycinea*, *maculicola*, *morsprunorum*, *papulans*, *phaseolicola*, *pisi*, *savastanoi*, *sesami*, *syringae*, *tagetis*, *theae* and *tomato*.

Comparative sequence analysis of appropriate genes should reflect continuity of nomenclature (Stackebrandt et al. 2002; Tindall et al. 2010). These genes should indicate relationships that correspond to that of the overall genome.

Anzai et al. (2000) undertook comprehensive comparative analysis of the 16S rDNA of *Pseudomonas* and demonstrated that the *P. syringae* group comprises of *P. amygdali*, *P. avellanae*, *P. caricapapayae*, *P. cichorii*, *P. ficuserectae*, *P. meliae*, *P. savastanoi*, *P. syringae* and *P. viridiflava*. The 16S rRNA being highly conserved, no discrimination within the group was achieved.

Multilocus sequence typing analysis of seven genes encoding sigma factor (*rpoD*), gyrase (*gyrB*), aconitate hydratase (*acnB*), phosphoglucosomerase (*pgi*), glyceraldehyde 3-phosphate dehydrogenase (*gap*) and phosphofructokinase (*pfk*) lying on the core genome were undertaken by Sarkar and Guttman (2004). Phylogenetic analysis revealed four major groups; three of these corresponded to the three groups of Sawada et al. (1999) and the fourth was a heterogenous group comprising of pathogens of monocots (rice, oats, etc.). Nearly 80 % of the total variation within the populations was strain specific and only 20 % of the variation was host specific. Some of the strains remained fairly stable genetically, e.g. eight soya bean (*glycinea*) pathogens collected over 13 years were genetically identical. On the contrary, even the strains isolated from the same host may show divergence, e.g. radish pathogen PmaKN91 clusters with bean pathogens in group III, while all other maculicola pathovars are in group I. Tomato pathogens fall in both group I and group II. Such variations are common in strains isolated from different host plants. The overall ecology of *P. syringae* strains is very similar, i.e. all are commensal and/or pathogens of the aerial plant surfaces and lack reliable biochemical and physiological distinctions that differentiate the four groups. Only a small number of core gene alleles are shared by the members of four groups. The noncore genes (e.g. associated with virulence genes such as type III effectors) support extensive horizontal gene transfer among strains. Thus *P. syringae* is a highly clonal organism. The factors outside the core genome play an important role in determining the host specificity. Genes coding for virulence factors such as type III effector proteins, toxins and resistance may be the key factors.

Multilocus sequence typing (MLST) of seven genes (*gapA*, *pgi*, *rpoD*, *gyrB*, *pfk*, *acnI*, and *cts*) of 60 pathovars of *P. syringae* species complex identified into five distinct phylogenetic clusters called phylogroups (Sarkar and Guttman 2004; Hwang et al. 2005). Phylogroup I mainly comprised of pathogens of tomato and brassica species such as radish and cabbage and *Psto* DC3000, pathogenic to tomato and *Arabidopsis*. Phylogroup II showed wide host specificity and included pathogens of pea, bean, corn, brown rice and wheat and some nonpathogenic strains. Group III included pathogenic strains of beans (pvs. *glycinea* and *phaseolicola*), tobacco and cucumber. Group IV contained the most basal clade of pathogens of monocots (rice, oats and onions) and group V comprised of strains which did not cluster with the other four groups of *syringae*. The strains PmaE54326 and PmaYm7930 are pathogenic to radish crops and diverged before the diversification of other species. Majority of the strains fall in groups I, II and III.

To further establish phylogenetic structures and relationship between phylogenomic groups based on MLST analysis of seven genes and genomospecies, all the 67 pathovar-type strains with defined host ranges were sequenced for *rpoD* gene, 578 nucleotide locus coding for sigma factor 70 (σ^{70}) (Parkinson et al. 2011).

The study revealed good correlation between genomospecies and phylogroup classification given by Sarkar and Guttman (2004). Within the phylogroups all the strains grouped together in major clades that were supported with high bootstrap values (at least 95 %). Two pathovars of *P. syringae* pv. *helianthi* and pv. *tagetis* grouped together with *P. carica papayae* were not represented in the earlier phylogenetic study and were identified as phylogroup VI (Parkinson et al. 2011). Three pathovars *P. syringae*, pv. *theae*, pv. *morsprunorum* and pv. *actinidiae*, under genome species 8 (*P. avellanae*) were grouped as a distinct subclade within phylogroup I. This data lends further support to the fact that *P. syringae* is a highly clonal organism.

Polyphasic approach to classification with phenotypic description does not yield coherent groups that can be named as specific species or correspond to genomic species (Young 2000; Weller et al. 2000). Even the genome sequence data cannot be exclusively used for classifying species ignoring the morphological, physiological, biochemical and other characteristics, as suggested by Lindström and Martínez-Romero (2005). Such an approach would be contrary to all previous taxonomic intentions (Stackebrandt et al. 2002; Tindall et al. 2010). Therefore an alternate approach requiring both phenotypic and molecular data may be considered for formal classification of species and genomovars.

11.3 Plant Pathogenesis: An Overview

Pseudomonas syringae are leaf-borne commensals of a large number of field crops, fruit trees, vegetables and garden plants. These as epiphytic colonisers can gain entry into leaf tissue, establish infection sites in the apoplast (intracellular spaces), multiply within the host tissue and produce disease symptoms. Thus their pathogenesis of the host plant is a multistep process delineated as (1) entry into plant cell/tissue, (2) overcoming the host resistance mechanisms and (3) evoking disease and producing symptoms using specific invasive strategies and molecules. Disease caused by *P. syringae* produces a variety of symptoms including cankers, leaf and stem spots, blight, soft rot and galls (Table 11.1). For success of the infection process, each of the three steps is essential and has an important role to play. Factors responsible for pathogenicity and virulence include secretion of a number of secondary metabolites such as phytotoxins, hormones, proteolytic enzymes, exopolysaccharides, ice nucleation activity and type III secretion system (TTSS), a mechanism similar to the one recognised in pathogens of human beings and animals. Important criteria that play a role in the entry of the pathogen into the host cell have also been defined and have a definite role to play.

The nature and structure of the toxins produced by different pathovars of *P. syringae* are highly variable and so are the symptoms of the disease. Major groups of toxins and their mode of action are listed in Table 11.2. Some of the phytotoxins have a small peptide nature; tabtoxin has a β -lactam structure and is a

Table 11.2 List of phytotoxins produced by *Pseudomonas* species and pathovars

Phytotoxin	Class	Producer
Coronatine	Polyketide	<i>P. s. pvs. atropurpurea, glycinea, maculicola, morsprunorum, tomato</i>
Corpeptin	Lipodepsipeptide	<i>P. corrugata</i>
Fuscopeptin	Lipodepsipeptide	<i>P. fuscovaginae</i>
Syringopeptins	Lipodepsipeptide	<i>P. syringae</i> pv. <i>syringae</i>
Tolaasin	Lipodepsipeptide	<i>P. tolaasii</i>
Viscosin	Lipodepsipeptide	<i>P. marginalis</i>
Syngomycin	Lipodepsinonapeptide	<i>P. s. pvs. syringae, aptata, atrofaciens</i> <i>P. fuscovaginae</i>
Tabtoxin	β -lactam	<i>P. s. pvs. tabaci, coronafaciens, garcae</i>
Rhizobitoxine	Vinylglycine	<i>P. andropogonis</i>
Persicomycin	Fatty acid	<i>P. s. pv. persicae</i>
Phaseolotoxin	Sulfodiaminophosphinyl peptide	<i>P. s. pv. actinidiae, phaseolicola</i>
Tagetitoxin		<i>P. s. pv. tagetis</i>

rare example among secondary metabolites of *Pseudomonas*. Production of the phytotoxins has been correlated to the presence of plasmids, and a number of plasmids coding for phytotoxins have been identified (Vivian et al. 2001).

11.3.1 Entry of *Pseudomonas syringae*

As already mentioned members of the genus *Pseudomonas* are commensals and occupy the plant leaf surface and other surface areas of plants. They primarily gain access to the cells and tissue either through the openings of the stomata or some physical injury on the plant leaf surface as a result of wind, dust, etc. Apart from this the flagellar movement of *Pseudomonas syringae* strains play an important role in establishment of infection as mutants defective in flagellar movement showed reduced degree of virulence in the host plant (Haefele and Lindow 1987; Ichinose et al. 2003). Tobacco plants sprayed with mutants *fgtI* and *fgtII* (defective in flagellin glycosyltransferase 1 and 2 genes) of *P. syringae* pv. *tabaci* 6605 (*Pta6605*) showed significantly reduced severity of disease symptoms as compared to the wild-type strain (Taguchi et al. 2006a, b). Glycosylation of the flagellin is important for stability of flagella, polymerisation of flagellar protein and motility and overall disease development. Cellular motility provides an advantage to the pathogen in terms of acquiring nutrients, avoiding toxic substances and unfavourable environments, finding host cells and spreading effectively for development of the disease (Nguyen et al. 2009; Taguchi et al. 2010a, b; Ichinose et al. 2013).

Apart from liquid swimming, motility flagella also provide surface swimming and swarming motility. The surface swarming motility also requires type IV pili

(T4P) responsible for twitching movement (Shimizu et al. 2003; Taguchi and Ichinose 2013). Both are required for the entry of the cells into the plant's apoplastic spaces. T4P-defective mutants also impair the host sensitivity response (HR) in non-host plants, e.g. *Arabidopsis*, and there is reduced expression of the *hrp* gene (hypersensitive response and pathogenicity gene). Glycosylation of pilin is essential for surface swarming motility and causation of disease. Environmental conditions of wetness and viscosity are important for flagellar and T4P-mediated motility. High humidity and low viscosity are required for flagellar movement and swarming involving flagella while twitching by T4P takes place in relatively dry and high-viscosity situations.

Besides this chemotaxis and aerotaxis play an important role in virulence. Strains of *Pto* DC3000 preferably move towards open stomata rather than closed stomata (Melotto et al. 2006, 2008a, b). A suitable chemical attractant may play a role in this. Cells of *R. solanacearum* are specially attracted by diverse amino acids and organic acids in the rhizosphere of tomato plants. Non-chemotactic mutants *cheA* and *cheW* showed reduced virulence on tomato plants (Yao and Allen 2006).

11.3.2 Plant Immune Response Against *P. syringae*

Plants have evolved an effective immune response mechanism (Abramovitch et al. 2006; Jones and Dangl 2006; Spoel and Dong 2012). Plants primarily respond by two types of immune signaling: the pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI). PTI is initiated with the detection of PAMPs by receptors located in the plasma membrane (PM) referred as PM-localised pattern recognition receptors (PRRs) (Boller and Felix 2009; Schweissinger and Ronald 2012; Spoel and Dong 2012).

PTI is suppressed by virulence factors such as type III effector proteins produced by pathogens. Plants have evolved an effector-triggered immunity (ETI) system to counter suppression of PTI. ETI is activated through recognition of effectors by a disease resistance (R) protein complex. The R proteins contain nucleotide-binding and/or leucine-rich repeat domains. While PTI prevents multiplication of nonpathogenic microbes, the ETI is necessary for prevention of disease by pathogens. The overall immune response to *P. syringae* infection involves callose deposition to strengthen cell wall at the infection site, antibacterial phytoalexins, reactive oxygen species (ROS), pH changes and restriction of nutrient release. ETI is generally accompanied by hypersensitive response (HR) at the site of infection leading to death of the cell. Accumulation of salicylic acid (SA), the defence hormone, is important for systematic resistance of the entire plant against *P. syringae*. This phenomenon is referred as systemic acquired resistance (SAR) (Vlot et al. 2009). In contrast to SA, the plant hormone jasmonate (JA) promotes *P. syringae* infection through an antagonistic relationship between SA and JA signaling (Zheng et al. 2012; Browse 2009).

11.3.3 Overcoming Resistance of Host Plant and Pathogenesis

Pseudomonas syringae shows a relatively narrow host range (Hirano and Upper 2000). The host specificity is observed at the level of pathovar host species and race host cultivars. The specificity is determined by the combination of the effector genes and the corresponding resistance genes.

Harpin protein is a unique TTSS effector which is secreted outside the bacterial cells and also elicits HR response in tobacco after entry into host cells (Baltrus et al. 2011; Lindeberg et al. 2006). Harpin binds the plant-cell membrane bilayer and forms an ion-conducting pore for release of nutrients and delivery of virulence factors (Lee et al. 2001). Pathogenic bacteria also produce effector proteins that are co-expressed with the *Hrp* secretion apparatus and are translocated into the plant-cell cytoplasm. More than 30 effector molecules have been identified in *P. syringae* pv. *tomato* DC3000 which play a role in overcoming host resistance by suppressing PAMP-triggered immunity and effector-triggered immunity (Guo et al. 2009) by inhibiting signaling mechanism.

Hop Z1a, an acetyltransferase, destroys plant microtubule networks and vesicles to block a plant secretory pathway (Lee et al. 2012). Apart from the *hrp* effector system, they produce a number of phytotoxins such as coronatine, syringolin A, syringomycin, syringopeptin, phaseolotoxin and tabtoxin (Bender et al. 1999). Coronatine is known to inhibit stomal closure. Jasmonic acid (JA) and salicylic acid (SA) signaling is antagonistic. SA signaling is necessary for effective plant defence against pathogens and is responsible by activation of JA and coronatine signaling.

Pseudomonas syringae pv. *savastanoi* (Psa) also produces the plant hormone indole acetic acid (IAA) which perturbs regulation of hormonal balance in the host cell and suppresses the plant defences. Colonisation and growth of bacteria in the apoplastic region is important for pathogenesis. The cells adhere to cell surface and secrete viscous compounds like exopolysaccharides (EPS) to form a biofilm. *Pseudomonas syringae* is known to produce alginate and levan (Laue et al. 2006). The cells in the biofilm are extremely resistant to antimicrobial agents.

Rapid generation of active oxygen species such as hydrogen peroxide and superoxide is a typical defence response. Short peptides flg22 and elf18 of the flagellin protein and harpins from *Pseudomonas syringae* induce rapid generation of hydrogen peroxide in tomato, *Arabidopsis* leaf tissue and tobacco cell cultures (Yoshioka et al. 2011).

11.4 Virulence Factor and Molecular Mechanism

Pathogenesis is a complex process of interplay of the pathogen and the host cell and the net outcome of the three steps listed above which involves the synthesis and secretion of proteins, enzymes, plant hormones, toxins, effector molecules, etc. to facilitate the process. Genome sequencing of *Psto* DC3000 has shown that genes coding for type I, II, III, IV, V and VI secretion systems are present in the genome of

P. syringae pv. *tomato* DC 3000 along with the twin-arginine transporter (Tat) system. Type II, type III and Tat secretion systems are important in terms of virulence (Cunnac et al. 2009; Lindeberg et al. 2008; Clarke et al. 2010).

The type II secretion system is responsible for secretion of two phospholipase C (Plc) PlcA1 and PlcA2 enzymes and transport of plant-cell-wall-degrading enzymes. Enzymes PlcA1 and PlcA2 are synthesised in the cytoplasm and transported to the periplasm by the Tat transport system and then transported by the type II secretion system to the extracellular space. The Tat transport system is highly conserved among gram-negative bacteria for export of about 60 different folded proteins into the periplasmic space. Important among these are phospholipases, amidases and amino transferases involved in virulence (Frobel et al. 2012; Palmer and Berks 2012).

The type III system (TTSS) is encoded by *hrp* (hypersensitive response and pathogenicity) and *hrc* (*hrp*-conserved) genes. The TTSS has a crucial role in pathogenesis and delivers a cocktail of type III effectors into the plant cell by a syringe-like supramolecular complex across the bacterial cell envelope (Butner and He 2009).

11.5 Type III Secretion System and Effectors (TTSS and TTSE)

The type III secretion system (TTSS) similar to gram-negative bacteria pathogenic to human beings and animals has been identified in *P. syringae* and is required for growth in the susceptible host and for the activation of plant defence mechanism in non-host plants, i.e. the hypersensitive response (HR). The phytotoxins may be host specific and exhibit the same specificity as the producing pathogen or non-host specific with a wider host range of activity than the producing pathogen. Generally the phytotoxins produced by *P. syringae* are non-host specific and cause symptoms on many plants even on those which cannot be infected by toxin-producing pathogens (Telgi et al. 2011).

A cluster of genes, *hrp* region (hypersensitivity response and pathogenicity), is conserved in phytopathogenic prokaryotes and affects the ability of the pathogen to induce hypersensitive response in non-host plants, pathogenicity in host plants and the ability to grow within or on the surface of plants. *Hrp* genes code for regulation and synthesis of TTSS.

The type III secretion system is one of the important virulence determinants of gram-negative pathogenic bacteria. The TTSS was first reported in *Yersinia*, a pathogen of humans and animals. Now it has been found in taxonomically diverse gram-negative bacteria, pathogenic to plants and animals, as well as some non-pathogenic plant-associated bacteria such as *Pseudomonas fluorescens*. The type III secretions system (TTSS) is responsible for pathogenicity of *P. syringae* by injecting virulence effector proteins into the plant cells. The TTSS genes in *P. syringae* were originally designated as *hrp* (hypersensitive response and pathogenicity) required for hypersensitive response (HR) on non-host plants and pathogenicity (or parasitism) on host plants. A subset of nine TTSS genes encoding

conserved core components, designated as *hrc* (*hrp* conserved), is essential for pathogenicity by injection of effector proteins into the plant cells. The needle-like structure translocates effector proteins designated as T3SEs into the cytoplasm of the host cell and thus modulates the host defence signaling.

As a pathogen *P. syringae* enters the leaves through stomata, multiplies for days in intracellular spaces and produces necrotic lesions. The *P. syringae* Hrp TTSS injects parasitic effector proteins into the plant cell. The genes encoding these proteins are designated as *avr* (avirulence) or *hop* (*Hrp* outer protein). The structural components of the TTSS are clustered into a 26 kb pathogenicity island called the *hrp/hrc* locus, consisting of nine genes showing homology to TTSS components of animal pathogens (*hrc* genes) and 17 plant pathogen-specific (*hrp*) genes including regulatory and accessory components that are secreted but not translocated into the host cell. The *hrp/hrc* cluster is flanked on one side by a conserved effector locus (CFL), which encodes three T3SEs present in all characterised strains. The other flank of the *hrp/hrc* cluster consists of an exchangeable effector locus (EEL) which codes for a unique complement of T3SE in each strain (Deng et al. 2003; Hogenhout and Bos 2011).

Computational and functional analysis has been used to identify nearly 60 different T3SE gene families and amount for their genetic diversity and ecological distribution. The number of T3SE families carried by each strain varies from three for the nonpathogenic *P. syringae* pv. *syringae* 642 (*Psy642*) to 38 for *P. syringae* pv. *avellanae*. Strains falling in phylogroup I carry at least 30 functional T3SE families, phylogroup II had 11–21 T3SE families and phylogroup III between 19 and 30. The role of the TTSS in *P. syringae* interactions with plants is complex and different strains may use alternate mechanisms that contribute to growth and development of symptoms. The sequenced genome of *P. syringae* pv. *tomato* DC 3000 contains 50 confirmed or putative TTSS effector genes, nearly 300 genes implicated in virulence and more than 800 genes that are not found in *Pseudomonas aeruginosa* and *P. putida*, and about 7 % of the genome as mobile genetic elements. This possibly accounts for variability and flexibility in interactions of *P. syringae* with plants (Lindeberg et al. 2006; Hogenhout et al. 2009).

Regions that have been acquired through horizontal gene transfer (HGT) often show altered GC ratio and flanking regions that contain direct repeats, insertion sequence (IS) elements, tRNA genes and genes for transposases. The exchangeable effector loci (EELs) of several strains of *P. syringae* have been shown to contain a variety of effector genes, pseudogenes and mobile genetic elements.

An important feature of the TTSS island of *P. syringae* is that it has a tripartite structure. The central *hrp/hrc* region is flanked by exchangeable effector loci (EELs) that can differ among strains of the same pathovar and by a conserved effector locus (CFL) that appears similar in divergent pathovars such as *syringae* and *tomato*. TTSS effector genes are distributed in clusters associated with mobile genetic elements on chromosomes and on plasmids in various strains of *P. syringae*. The sequence of EELs showed that the region between *hrpK* and tRNA^{leu} contains at least one effector gene (or effector pseudogene) and in many cases mobile genetic elements. Secondly several of the effector genes are widespread among

P. syringae strains. Thirdly the deletion mutation in the EEL region does not suggest any universal function to this region. Finally analysis also suggests that *avrPphE* was acquired by *P. syringae* after *hrpK* and the rest of the *hrp* system but before divergence of the host-specific pathovars (O'Brien et al. 2011; Ichinose et al. 2013). Mosaic pathogenicity islands have been observed in other pathogens. The presence of mobile genetic elements and sequences such as effector genes which are associated with plasmids and *Is* elements can promote recombination in regions such as *Hrp* pathogenicity islands within *EEL* of *P. syringae*.

11.5.1 Structure of TTSS

Four polycistronic operons coding for TTSS protein components are inducible by HrpL and RspL. Nine of these proteins representing core components are highly conserved in the TTSS and play a role in export of proteins across the inner membrane and the outer membrane. These core Hrc proteins share sequence similarity with flagellar component except one, the Hrc C which belongs to the secretion family of outer membrane porins. TTSSs are characterised by (1) host-contact-mediated induction, (2) energy requirement for protein secretion, (3) -secretion-regulated expression of secreted proteins and (4) dedicated chaperones for some secreted proteins (Blocker et al. 1999).

Conserved Core of TTSS in the Bacterial Cell Envelope TTSSs of mammalian pathogens form a stable structure resembling a flagellar basal body referred as needle complex. This needle complex consists of a short, <100 nm-long, 8 - nm-diameter needle inserted in a 30 nm-diameter complex that traverses (Blocker et al. 2001). In contrast to the needled complex, the TTSS of *P. syringae* produces a filamentous appendage called Hrp pilus. This is approximately of a same diameter and several micrometres in length.

The key export apparatus is a triplet of highly conserved proteins Fli P/Q/R (cf. Hre R/S/T) similar to F₀F₁-ATPase (ATP 5/8/6). Membrane proteins FlhA and FlhB (HreV and HrU) are also conserved in most of TTSSs although *Hre V* is absent in *P. fluorescens*. Protein Fli Y (Fli N + Fli M) is present in both *P. syringae* and *P. fluorescens* as two proteins, HrcQ_A and HrcQ_B, rather than a single protein in most of other TTSSs.

Specialised Extracellular Components of Hrp TTSS In *P. syringae* two extracellular components have been identified: the *HrpA*-encoded Hrp pilus and harpins. Hrp pilus plays an essential role in effector secretion and translocation. HrpA and harpins are secreted in abundance as compared to effectors. Hrp pilus is a surface appendage measuring 8 nm in diameter and several micrometres in length. New HrpA subunits are added at the distal end. HrpA appears to be a conduit for transport of TTSS-secreted proteins to the plant-cell cytoplasm. HrpA pilin subunit encoded in the *Hrp/hrc* gene cluster is an operon that also encodes HrpZ and HrcJ.

Comparative studies have shown that HrpA is the most variable component of the system, e.g. HrpA protein of *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *syringae* 61 share 31 % identity. Many of the TTSS effector proteins depend upon chaperones. The pilus proteins HrpA (*P. syringae* and *E. amylovora*), HrpY (*R. solanacearum*) and HrpE (*X. campestris* pv. *vesicatoria*) don't have any significant sequence homology but have common physicochemical features, e.g. size (8.7–11.3 kDa), consist of α -helices, similar hydrophobicity characteristic, etc. It is suggested that an effector passes through the Hrp pilus in an unfolded stage as the pilus has an internal diameter of 2 nm. HrpZ and HrpW harpins are another class of protein that travels through the Hrp pilus. Harpins are glycine rich and cystine lacking and possess stable HR elicitor activity.

The type III effector proteins of *P. syringae* are grouped as (1) intracellular type III effectors which are directly transported from the bacterial cell to the cytosol of the plant cell and (2) extracellular type III effectors comprising of *hrpA*-encoded Hrp pilus protein and harpins. Hrp pilus plays an essential role in the effector secretion and translocation process for effective delivery of the effectors in the host cell cytoplasm. Thus the major virulence function of intracellular TTSS effectors is the suppression of various plant defences including basal resistance, gene to gene resistance and non-host resistance. More than 30 different types of effector molecules have been identified in *P. syringae* Pto DC3000 which largely act as enzyme inhibitors and suppress PAMP-triggered immunity and effector-triggered immunity (Guo et al. 2012). Harpins are considered as helpers in the delivery of the effector molecules. Besides these, the TTSS clusters of *P. syringae* and *P. fluorescens* code several other proteins whose functions are not yet elucidated but are important for TTSS function (Table 11.3). Comparative studies have shown that HrpA is a most variable component of the system of HrpA proteins of *P. syringae* pv. *tomato* DC3000 and *P. syringae* pv. *syringae* 61 shares only 31 % identity (Deng et al. 2009; Hogenhout and Bos 2011).

11.5.2 Regulation of TTSS

Pseudomonas syringae up-regulates its *hrp* gene expression after inoculation into the plant tissue. An acidic minimal medium containing fructose as carbon source induces *hrp* genes in *P. syringae* pathovars. TTSS expression is subject to catabolite repression as the TCA cycle intermediates and L-glutamate suppress *hrp* induction. Some plant-specific signals have been reported to be involved in TTSS expression in *Ralstonia* and *Rhizobium* and some *Pseudomonas* *hrp* genes (Tang et al. 2006).

Transcription of the *hrp* locus is regulated by proteins coded within the TTSS gene cluster. The σ^{54} , EBP's HrpR and HrpS activate transcription of HrpL from an RpoN (σ^{54})-dependent promoter. RpoN is required for TTSS expression and virulence in *P. syringae* pv. *maculicola*. HrpL then activates expression of other components of the Hrp secretion apparatus and Hrp-secreted effectors in *P. syringae*.

Table 11.3 Avr proteins and type III secreted/effector proteins identified in *P. s. pvs. tomato* and *maculicola* (Preston 2000)

Name	Origin	Location	Description	Cellular location, phenotype and function
HrpZ	<i>P. s. pv. tomato</i> DC3000 ^a	Chromosome—hrp/hrc gene cluster	370 amino acids (36.5 kDa) Glycine-rich 'harpin'	Apoplast/cell wall, putative type III translocation Accessory protein. Purified protein elicits HR in tobacco Not required for HR or pathogenicity
HrpW	<i>P. s. pv. tomato</i> DC3000 ^a	Chromosome—conserved effector locus (CEL)	425 amino acids (42.9 kDa) Harpin-like N-terminal, pectate lyase-like C-terminal	Apoplast/cell wall, putative type III translocation accessory protein. Binds pectate. Purified protein elicits HR in tobacco Not required for HR or pathogenicity
AvrA	<i>P. s. pv. tomato</i> PT23	Chromosome?	907 amino acids (100 kDa) AvrA allele in <i>P. s. pv. glycinea</i>	Host cytoplasm? Confers avirulence on soybean. Mutant reduced in virulence
AvrD	<i>P. s. pv. tomato</i> PT23	Plasmid pPT23B	311 amino acids (34 kDa) AvrD alleles in <i>P. s. pv. glycinea</i> and other <i>syringae</i> pvs.	Synthesis of syringolide elicitors Rpg4 (R)—soybean. Confers avirulence on soybean. No effect on virulence
AvrE	<i>P. s. pv. tomato</i> DC3000 & PT23	Chromosome—CEL	1795 amino acids (195 kDa) Homologous to DspE/DspA from <i>Erwinia</i> spp. Secreted in culture	Apoplast—plasma membrane? Confers avirulence on soybean PT23 mutant reduced in virulence, DC3000 mutant no effect on virulence. No effect on HR
AvrPto	<i>P. s. pv. tomato</i> DC3000; JL 1065	Chromosome	164 amino acids (18.3 kDa) Acylation motif	Host cytoplasm—membrane Pto (R)—tomato. Interacts with Api proteins. Confers avirulence on soybean and tomato cultivars. Enhances virulence and necrosis on susceptible host
AvrRpt2	<i>P. s. pv. tomato</i> JL 1065	Probably chromosome	256 amino acids (28.2 kDa) Hydrophilic protein	Host cytoplasm. RPS2 (R)— <i>A. thaliana</i> . Confers avirulence on <i>A. thaliana</i> , bean and soybean. Transgenic expression causes browning of <i>A. thaliana</i>
AvrRpm1/ AvrPmaA1	<i>P. s. pv. maculicola</i> M2	Plasmid (chromosomal alleles in some strains)	220 amino acids (24 kDa) Acylation motif AvrPpiA1 allele in <i>P. s. pv. pisi</i>	Host cytoplasm—plasma membrane. RPM1 (R)— <i>A. thaliana</i> . Confers avirulence on <i>A. thaliana</i> , soya bean, pea (R2), bean (RN1/RN2). Required for full virulence on <i>A. thaliana</i>
EEL ORF1	<i>P. s. pv. tomato</i> DC3000	Chromosome—exchangeable effector locus (EEL)	466 amino acids (50 kDa) No similarity to known proteins	Hrp-secreted protein. Function unknown
CEL ORF1	<i>P. s. pv. tomato</i> ^a DC3000	Chromosome—CEL	495 amino acids (54 kDa) Similar to <i>E. coli</i> MltD Lysozyme-like domain	Type III accessory protein? May facilitate insertion of type III system through the peptidoglycan layer. Mutants have no obvious phenotype

^aProbably common to many, if not all, strains of *P. syringae*. (R) Corresponding resistance gene, where known

Some environmental factors such as nutrient status and pH play an important role in transcriptional regulation of *hrpR*, *hrpS* and *hrpL* by introducing conformational changes in these regulatory proteins. The regulatory mechanism of *P. syringae* pv. *phaseolicola* significantly differs from other *P. s.* pathovars (Schuster and Grimm 2000).

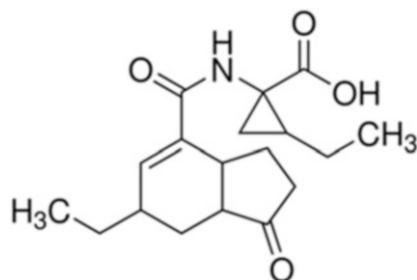
11.6 Toxins and TTSS Independent Virulence Factors

The phytotoxins produced by *P. syringae* generally induce chlorosis (coronatine, phaseolotoxin and tabtoxin) or necrosis (syringomycin and syringopeptin). The activity of some of the phytotoxins may be manifested only at the biochemical level. Phytotoxins may not be directly involved in pathogenicity but function as virulence factors for *P. syringae* and enhance severity of the disease by enhancing movement of bacteria in plant tissue, increasing lesion size and multiplication of the pathogen in the host.

11.6.1 Coronatine

11.6.1.1 Structure and Biosynthesis

Coronatine (COR) is produced by a number of pathovars of *P. syringae*, e.g. *atropurpurea*, *glycinea*, *maculicola*, *morsprunorum* and *tomato* (Bender et al. 1999). COR has an unusual chemical structure and consists of two components, viz. (a) the polypeptide coronafacic acid (CFA) and (b) coronamic acid (CMA). Coronamic acid is an ethylcyclopropyl amino acid derived from isoleucine. Coronatine shows structural and functional similarity to methyl jasmonate, a plant hormone which activates the jasmonate (JA) involved in activation of the plant defence against herbivores and certain pathogens. Coronatine has a dual function; on one hand it activates JA signaling response and on the other it suppresses salicylic acid (SA)-dependent plant defence mechanism.



Structure of Coronatine

The generalised biosynthetic pathway is based on ^{13}C -labelled substrates. CFA is synthesised from one molecule of pyruvate, one molecule of butyrate and three molecules of acetate. The pyruvate is converted into α -ketoglutarate and then

α -ketoglutarate serves as the starting point for polyketide coronafacic acid (CFA) assembly. Mitchell et al. (1995) identified a cyclopentenone compound, 2 [1-oxo-2cyclopenten-2-ylmethyl]-butanoic acid (CPE), which may function as an intermediate or shunt product of the CFA biosynthetic pathway.

This hypothesis postulates deamination of glutamate to α -ketoglutarate through decarboxylation. The decarboxylation reaction yields succinic semialdehyde (SSA) that would be converted into its COA ester before serving as a starter unit for polyketide assembly. The isolation of CPE from *P. syringae* suggests that a cyclopentenone is formed during biosynthesis of CFA. L-alloisoleucine has been demonstrated as a more immediate precursor of coronamic acid (CMA) synthesis than isoleucine (Parry et al. 1991). The pathway involves isomerisation of isoleucine to form alloisoleucine and cyclisation of alloisoleucine to form CMA. The final step in the COR synthesis involves the ligation of CFA and CMA by an amide linkage bond formation by coronafacate ligase coded by *cfl* (Liyanage et al. 1995).

11.6.1.2 Biological Effect and Mode of Action

Tissues treated with COR or infected with COR-producing *P. syringae* show intense spreading chlorosis attributed to loss of chlorophylls a and b in tomato and tobacco plants. The chloroplasts in such plants are generally smaller and have a reduced amount of starch deposits. ELISA and immunolocalisation studies indicated that COR associates with chloroplasts in tomato leaves and probably acts by inducing the expression of chlorophyllase, the first enzyme in the chlorophyll degradation pathway.

Apart from chlorosis, COR also affects anthocyanin production, alkaloid accumulation, ethylene emission, tendril coiling, root elongation and hypertrophy. COR also induces volatile production and accumulation of proteinase inhibitors. In monocots and grasses, COR may be associated with apoptotic cell death and accumulation of flavonoid phytoalexins.

Coronatine plays an important role in overcoming stomatal defence by inhibition of stomatal closure, an important host defence mechanism. Normally stomata closes within one hour of exposure to bacterial suspension and limits the entry of bacteria into the leaf tissue. Stomata closure is triggered by PAMPs such as flg22 (a peptide of bacterial flagellin) and lipopolysaccharide (LPS) as an output of PTI system (Melotto et al. 2008a, b). Besides overcoming negative effect of stomatal closure on pathogenesis, coronatine also helps in multiplication and persistence of bacteria in the apoplast (Melotto et al. 2006).

Coronatine does promote development and production of symptoms which represent the final phase of pathogenesis and cause crop loss and marketability. Plants treated with coronatine develop chlorosis while coronatine-deficient mutants show reduced chlorosis and necrosis (Brooks et al. 2005). Expression of the nuclear gene STAYGREEN (SGR or Mendel's I locus) is critical as SGR codes for a protein involved in chlorophyll degradation.

COR has a structural similarity with jasmonic acid (JA) and thus functions as an analogue of JA and related signaling compounds, e.g. methyl JA (meJA) and 12-oxo-phytodienoic acid (12 OPDA), a precursor of JA/MeJA biosynthesis.

JA and MeJA are plant growth regulators induced in response to biological stress by herbivores and certain fungi. When the plasma membrane is damaged, the linolenic acid is converted to OPDA by the action of lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC). OPDA is further modified by reductase and three β -oxidation steps leading to formation of JA. OPDA, JA and other octadecanoid molecules trigger gene expression by acting upon various molecular targets (Katsir et al. 2008).

In addition JA plant defence in response to microbial attack is regulated by salicylic acid (SA) and ethylene production. SA plays a key role in defence mechanism. Even the exogenous application of salicylic acid enhances resistance to a broad range of pathogens Ryals et al. (1996). SA is required for rapid activation of a defence response and contains the growth of pathogens.

Plant mutants that lack the ability to produce SA and transgenic plants with the SA-degrading enzyme salicylate hydroxylase (NahG) gene *nahG* show higher susceptibility to *P. syringae* (Wildermuth et al. 2001). The inhibitory effect of SA on JA signaling has been studied in tomato and *Arabidopsis thaliana* and the primary mode of interaction appears to be mutual antagonism. The *coil* (JA-insensitive) lines of *A. thaliana* show elevated resistance to *P. syringae* infection and impaired multiplication of bacterial population as well as symptom development. Also, increased levels of SA and hyper-expression of the SA-dependent pathogenesis-related gene PR-1 were observed in *coil* plants inoculated with *P. syringae* pv. *tomato* DC 3000. These studies imply a connection between COR and modulation of the SA pathway. The ability of the pathogenic bacteria either to inhibit or delay SA-dependent response is an important trait as this provides the pathogen an opportunity to colonise the host tissue. The overall mechanism suggests that COR may promote virulence by targeting the JA signaling pathway in tomato with resulting antagonism of the SA pathway (Zhao et al. 2003; Xin and He 2013).

Recently, three *NAC* (*NAM*, *ATAF1,2*, *CUC2*)- family transcription genes have been identified, and mechanism of COR-activated signaling cascade has been elucidated (Zheng et al. 2012). The transcription factor MYC2 directly activates the expression of *ANAC019*, *ANAC055* and *ANAC072* which then differently regulates the expression of *ICS1*, *BSMT1* and *SAGT1*. Repression of the SA biosynthesis gene *BSMT1* results in net reduction in the accumulation of salicylic acid (SA) and thus compromises resistance of the host to the pathogen.

Thus the gene products *ANA019*, *ANAC055* and *ANAC072* have a dual role as having transactivation activity and also as transcriptional suppressors, binding the *ICS1* promoter and repressing its expression. The dual transcriptional capability may be achieved by interacting with different transcriptional components.

It is clear that COR acts by suppressing host defence mechanism by inhibiting SA accumulation and promoting bacterial multiplication inside the apoplast. COR also induces symptom development, i.e. chlorosis, by inducing SRG (STAYGREEN or Mendel's locus mutant) that codes for a protein involved in chlorophyll degradation (Armstead et al. 2007; Mecey et al. 2011).

11.6.1.3 Genetics and Regulation of COR Production

Phytotoxin coronatine is produced by a number of pathovars of *P. syringae* including pvs. *alisalensis*, *atropurpurea*, *glycinea*, *maculicola*, *morsprunorum* and *tomato* which cause disease in broccoli, ryegrass, soya bean, crucifers, *Prunus* spp. and tomato, respectively. The COR gene cluster has been reported to be plasmid borne in pvs. *atropurpurea*, *glycinea*, *morsprunorum* and *tomato*, while it is encoded on the chromosome in strains of the pathovar *maculicola*. Some exceptions do occur to this general rule. The COR gene have been shown to be associated with large, 80–100 kb, plasmids. The COR plasmids contain the origin of replication (*oriV*) and stability gene (*par*) identified in pOSU900. The *oriV* and *par* loci of pOSU900 are widely distributed in *P. syringae* (Sesma et al. 1998).

COR biosynthesis in *P. syringae* pv. *glycinea* PG4180 which infects soya bean is encoded on a 90 kb plasmid designated as p4180A. Two regions in the COR biosynthetic cluster contained structural genes for CMA and CFA synthesis; these were separated by a 3.4 kb regulatory region. Detailed studies indicated that a COR gene cluster in *P. syringae* pv. *glycinea* PG 4180 consists of six transcripts, *cmaU*, *cmaTBA*, *corP*, *corS*, *corR* and *cFA* (Budde et al. 1998).

The nucleotide sequence of a 6.9 kb region comprising a CMA biosynthetic gene cluster revealed the presence of four genes, viz. *cmaA*, *cmaB*, *cmaT* and *cmaU*. These genes cluster into two transcripts; one transcript is monocistronic with the *cmaU* gene, while the other is polycistronic with three cotranscribed genes, *cmaA*, *cmaB* and *cmaT*. The *cmaB* gene showed extensive homology to the *syrB2* gene encoding an enzyme required for syringomycin synthesis. The deduced amino acid sequence of the *cmaT* product suggested that it functions as a thioesterase (TE) which indicated that thiotemplate mechanism plays role in CMA biosynthesis. The *cmaU* sequence analysis has indicated relatedness to threonine efflux proteins and may be involved in transport of CMA or COR to the external environment.

The CFA region is contained in a single 19 kb transcriptional unit. Polyketides show a high degree of structural and functional diversity. Polyketide biosynthesis is similar to the fatty acid biosynthesis. As a first step, acetyl COA condenses to form malonyl-COA, a chain extender unit. The reaction is catalysed by ketosynthase (KS) to produce an enzyme-bound β -keto thioester. Then the ketogroup is reduced to a methylene group in a three-step reaction catalysed by ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER). The intermediates are bound to the acyl carrier protein (ACP). The CFA transcript contains ten discrete ORFs. *cfa1*, *cfa2* and *cfa3* are related to ACP, fatty acid DH and β -ketoacyl synthase, respectively. Gene products of *cfl* and *cfa5* are similar to acyl-COA ligases, products of *cfa6* and *cfa7* show similarity to DEBS (6-deoxyerythronolide B synthase), and a PKS is involved in erythromycin synthesis, indicating participation of multifunctional PKSs. *cfa8* and *cfa9* show relatedness to crotonyl-COA reductases and TEs, respectively.

The enzyme coronafacate ligase which catalyses ligation of CFA and CMA via an amide bond is encoded by a 1.4 kb gene designated as *cfl*. This gene also forms part of the CFA transcript.

In *P. syringae* pv. *tomato* DC3000, a pathogen of tomato, *Brassica* spp. and *Arabidopsis thaliana*, the COR genes are encoded chromosomally, and the structural genes of CMA and CFA are separated by a 26 kb region. Both clusters are bound by *Is* elements indicating that these may be mobile in nature and may be present on the chromosome or plasmid. These show high degree of homology (90–98 % nucleotide identity) to that of *P. syringae* pv. *glycinea* PG4180. Using in vivo expression technology, it has been shown that approximately 15 % of genes induced during infection are localised in CFA operon and included *cfl*, *cfa1*, *cfa7*, *cfa8* and *cfa9*. Malic acid, citric acid, shikimic acid and quinic acid have been reported to stimulate COR production and are normally present in leaf extracts and apoplastic fluids of tomato (Li et al. 1998).

Nutrition and environmental factors play an important role in COR production by *P. syringae* pv. *glycinea* PG4180. The effect of temperature was highly significant with maximum production at 18 °C and negligible at 30 °C; however, the growth of *P. syringae* pv. *glycinea* PG4180 was unaffected of temperature ranging from 14 to 30 °C. Rohde et al. (1998) showed that COR production was thermoregulated in pvs. *atropurpurea*, *maculicola*, *morsprunorum* and *tomato* and may be extrapolated as common regulatory control for COR production. Temperature regulates the production of CFA and CMA at the transcriptional level.

Sigma 54 (σ^{54}), encoded by *rpoN*, is an absolute requirement for COR production and *cor* gene expression in *P. syringae* pv. *maculicola* ES4326 and pv. *glycinea* PG4180. The regulatory region which controls both CFA and CMA production contains three regulatory genes, *corP*, *corS* and *corR* (Ullrich et al. 1995). The deduced amino acid sequences of *corP* and *corR* indicated relatedness to response regulators that function as two component regulatory systems, and the *corS* gene product showed sequence similarity to histidine kinases which act as environmental sensors. The response regulators control the adaptive response in twin component regulatory systems and are characterised by an N-terminal receiver domain which functions as a phosphorylation site and a C-terminal effector domain with a DNA binding, the helix-turn-helix (H-T-H) motif. Both these are strongly conserved in *corR*; however, *corP* contains the highly conserved receiver domain (at least two aspartate residues and a conserved lysine) but lacks the H-T-H motif. The N-terminal receiver domains of CorR and CorP are identical and probably share specificity for the same phosphodonor protein.

Environmental factors such as carbon source and amino acid availability and osmolarity affected COR production in PG4180. Glucose level and selected carbon and amino acid sources affect expression of the *cfl* and *comaA* operons. Genes were strongly repressed by xylose and fructose and amino acids isoleucine and valine.

11.6.2 Lipodepsipeptide Toxins: Syringomycins and Syringopeptins

Pseudomonas syringae strains produce lipodepsipeptide phytotoxins such as syringomycins and syringopeptins which contribute towards virulence. These are synthesised using non-ribosomal peptide synthases and polyketide synthases.

Besides these antimetabolite toxins such as phaseolotoxin, tabtoxin and recently identified mangotoxin isolated from *P. syringae* pv. *syringae* strain UMAF0158-A which causes apical necrosis of mango have been identified. These toxins specifically inhibit the activities of enzymes of arginine and ornithine synthesis.

Biosynthesis loci for two additional *P. syringae* toxins have been identified. First of these the *syf RABCD* codes for gene products responsible for production of six lipodepsipeptide compounds termed syringofectin *SyrA* to *SyrF*. They are linear and play a role for surfactant activity and swarming motility. Second is mangotoxin responsible for apical necrosis of mango (Berti et al. 2007; Lindeberg et al. 2008).

Syringomycins and syringopeptins are necrosis-inducing lipopeptide phytotoxins produced by *Pseudomonas syringae*. These were originally isolated from *P. syringae* pv. *syringae*. However, most of the other pathovars have been reported to produce these. The cyclic peptides of syringomycins and syringopeptins target host cell membrane and induce pore formation leading to leakage of electrolytes and efflux of K^+ from plant cells. Syringomycins and syringopeptins show structural and functional similarity and their gene clusters are located adjacent to each other but are physically distinct (Bender et al. 1999).

11.6.2.1 Structure of Syringomycins

Syringomycins are lipodepsinonapeptide phytotoxins consisting of a hydrophobic-3-hydroxy-carboxylic acid tail and a charged cyclic peptide head comprising of nine amino acids (Fig. 11.2). On the basis of the fatty acid tail possessing decanoic, dodecanoic or tetradecanoic acid, these are referred as SRA, SRE and SRG. The 3-hydroxy fatty acid tail is attached to serine through an amide bond. The ester

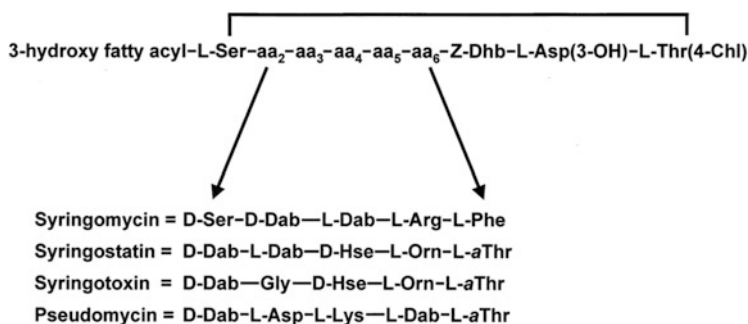


Fig. 11.2 Structure of syringomycin, syringostatin, syringotoxin and pseudomycin differs with respect to amino acids at positions 2–6. The 3-hydroxy fatty acid group is a derivative of decanoic acid (syringomycin), dodecanoic acid (syringomycin and syringostatin), tetradecanoic acid (all the four) or hexadecanoic acid (pseudomycin); some forms of pseudomycin are acylated by 3,4-dihydroxytetradecanoic acid or 3,4-dihydroxyhexanoic acid. Abbreviations for amino acids are Asp(3-OH), 3hydroxyaspartic acid; Dab, 2,4-diaminobutyric acid; Dhb, 2,3-dehydroaminobutyric acid; Hse, homoserine; Orn, ornithine; Thr(4-Chl), 4-chlorothreonine; and αThr, allothreonine

linkage between the N-terminal serine and C-terminal residue, 4-chlorothreonine, forms a macrocyclic lactone ring. The cyclic peptide comprises of a number of rare amino acids, viz. 2,3-dehydroaminobutyric acid, 3-hydroxyaspartic acid, 4-chlorothreonine and D-isomers of serine and 2,4-diaminobutyric acid. For their biological activity, chlorination of threonine is essential (Grgurina et al. 1993, Scaloni et al. 1994). The syringomycin results in pore formation of the host cell lipid bilayer thereby increasing the efflux of K^+ , H^+ and Ca^{++} that are deadly to the cells. Pore formation and lysis occur at threshold level of 50 ng/ml of syringomycin. Syringomycin monomers aggregate to form pore complexes. A channel is formed by at least six molecules of syringomycin. The lipophilic portion lies in the core of the bilayer, while the hydrophilic peptide head resides close to the surface of the membrane. The channel radius is ~ 1 nm which is comparable to alpha-hemolysin, a cytolytic protein produced by *E. coli*.

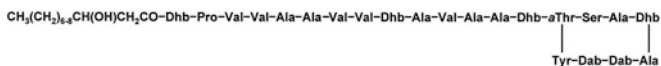
11.6.2.2 Structure of Syringopeptins

Syringopeptins contain a peptide moiety of 22–25 amino acids. The peptide is attached to a 3-hydroxydecanoic or 3-hydroxydodecanoic acid, thus forming syringopeptin A and syringopeptin B, respectively. They contain a number of hydrophobic amino acids, many of which are in D-isomeric form. Three isoforms of syringopeptin 22 (SP₂₂) vary in amino acid composition and two isoforms of syringopeptin 25 (SP₂₅) differing in their C-terminal amino acid have been characterised from *Pseudomonas syringae* pv. *syringae* (Fig. 11.3). All isomers are conserved to form a lactone ring via an ester bond between C-terminal residue and the conserved *allo*threonine (*athr*). Diversity in the structure of syringopeptins has been reported in different strains of *P. syringae* pv. *syringae* (Isogai et al. 1995).

11.6.2.3 Cytotoxicity and Mode of Action

These nonspecific and necrosis-inducing phytotoxins insert into the plant cytoplasmic membrane resulting in transmembrane flux of K^+ , H^+ and Ca^{++} and death of the plant cell due to disruption of the electrical potential across the plant-cell membrane. The toxins are highly effective and syringopeptins exhibit greater toxicity due to their relatively larger size. Virulence assays using *P. syringae* pv. *syringae*

SP22



SP25

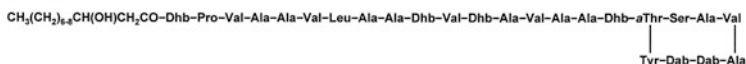


Fig. 11.3 Structure of syringopeptin forms SP22 and P25. The fatty acid can be either 3-hydroxydecanoic acid or 3-hydroxydodecanoic acid. Abbreviations for the unusual amino acid are Dab, 3,4-diaminobutyric acid; Dhb, 2,3-hydroaminobutyric acid; and αThr , *allo*threonine. D-amino acids are common in both SP22(13-22) and SP25(15-25) residues

mutants in syringomycin synthetase (*syrB1*) and syringopeptin synthetase (*sypA*) in cherry fruits showed 26 % and 59 % reduction in virulence, respectively, while the double mutants *sypA* and *sypB* showed up to 76 % reduction. This was comparable to the *syrD* mutant BR105 which is deficient in secretion of both syringomycin and syringopeptin. Three different modes of action have been reported for syringomycin and syringopeptin:

1. Pore formation
2. Biosurface activity
3. Antimicrobial activity

Pore Formation Biophysical analysis has shown that monomers insert into the membrane lipids and quickly aggregate to form a pore. The syringomycin pores are formed by aggregation of six (± 1) monomers and a hydrophilic portion lies on the cell surface and hydrophobic portion of the lipid bilayer. The syringomycin-induced pores could coalesce to form larger channels and the syringomycin pore radius could vary from 0.6 to 1.7 nm (Dalla-Serra et al. 1999).

The syringopeptin-induced pores are water-filled pores with the hydrophobic residues of the phytotoxin lining the pores. Pore formation may require 3–5 (± 1) monomers. The two types of pores may act synergistically but there is no evidence that pores are composed of a combination of two types of phytotoxins.

Pore formation is influenced by sterols. Both syringomycin and syringopeptin can form pores in artificial membranes lacking sterols, but addition of cholesterol (component of animal membrane) or ergosterol (component of fungal membrane) to the lipid bilayer increases the pore-forming activity of syringomycin. Ergosterol also enhances the binding of syringomycin to the cell. On the contrary, the syringopeptin does not require sterols but an increase in pore-forming activity was observed in the presence of stigmaterol (a component of plant membrane). Addition of ergosterol had an adverse effect on syringopeptin pore formation.

Biosurfactant Activity Both syringopeptin and syringomycin show biosurfactant activity and reduce the interfacial water tension. Their surface activities are at much lower concentrations as they show critical micelle concentration of approximately 1.2 mg/ml in the case of syringomycin and 0.8 mg/ml in the case of syringopeptin. Reduction in surface tension enables the bacteria to spread across plant surfaces (Dalla-Serra et al. 1999; Hutchison and Gross 1997).

Antimicrobial Activity Besides phytotoxic effects, syringomycin also exhibits antifungal activity against *Geotrichum candidum* and the yeast *Rhodotorula pilimanae*. Syringopeptin is inhibitory to *Bacillus megaterium* and the fungus *Botrytis cinerea*. Some of these organisms are highly sensitive and *B. megaterium* is used as an indicator strain in bioassays (Sorensen et al. 1996).

11.6.2.4 Biosynthesis of Syringomycin and Syringopeptin

Biosynthesis of syringomycin and syringopeptin takes place through a non-ribosomal thiotemplate mechanism catalysed by multifunctional enzymes called peptide synthetases. The peptide synthetases carry out activities of repeated amino acid activation, condensation, amino acid adenylation and thiolation involving large proteins, 470 kDa or larger in size, with lipodepsinonapeptide production (Xu and Gross 1988). Syringomycin peptides contain non-proteinogenic amino acids and D-amino acids.

Syringomycin Synthesis Genes for syringomycin synthesis are carried on a ~58 kb segment on the chromosome of *P. syringae* pv. *syringae* B310D. Two genes, *syrE* and *syrB1*, comprising of 32.2 kb encode peptide synthetases. Biosynthesis of syringomycin occurs in a nonlinear manner and the *syrB1* gene is located upstream of *syrE*. The *syrE* gene encodes eight amino acid activation modules which contain domains for condensation, aminoacyl adenylation and thiolation. The aminoacyl adenylation domain controls substrate specificity for amino acid (SyrE-M1), and L-serine has been identified as the first amino acid activated by SyrE at the N-terminal.

Molecular genetic evidence for thiotemplate mechanism of syringomycin synthesis came from the analysis of *syrB1* gene coding for the amino acid activation module of the peptide synthetase. The *syrB1* protein sequence revealed six core sequences typical of amino acid-activating molecules similar to the ones involved in synthesis of peptide amino acids.

The syringomycin (Syr) gene cluster comprises ~37 kb locus on the chromosome of *P. syringae* pv. *syringae* B301D. The *syr* gene cluster comprises of six ORF-encoding proteins involved in synthesis (*syrB1*, *syrB2*, *syrC* and *syrE*), secretion (*syrD*) and regulation (*syrP*) of syringomycin. The *syrE* ORF is enormous in size (28.4 kb) and codes for a 1039 kDa peptide synthetase which contains eight amino acid activation modules. Each of the amino acid activation modules contains a domain for elongation, adenylation and thiolation (Guenzi et al. 1998).

Syringopeptin Synthesis The *syp* gene cluster has a size of ~85 kb and contains three genes *sypA*, *sypB* and *sypC* measuring 16.1, 16.3 and 40.6 kb, respectively. Each one of these genes codes for peptide synthetase containing 5, 5 and 12 amino acid activation module of the 22 amino acid sequence of syringopeptin (Scholz-Schroeder et al. 2001, 2003).

Synthesis of syringopeptin takes places by non-ribosomal peptide synthesis (NRPS). The *sypA* contains the activation module for first five amino acids. A condensation domain is involved in aminoacyl transfer of 3-hydroxy fatty acid to the activated Dhb attached to SypA-M1. This follows the activation and incorporation of the next four amino acids into syringopeptin with the translocation of growing peptide from one module to the next. Then the N-terminal contestation domain of *sypB-M6* interacts with *syp-M5* for transferring the growing peptide to

sypB for the stepwise incorporation of the next five amino acids. The N-terminal condensation domain of *sypC*, *sypC*-M11 interacts with SypM10 to transfer growing peptide to SypC. The growing peptide is then translocated from SypC-M11 to Syp-M-22 and the remaining 12 amino acids are activated and incorporated in syringopeptin.

Syringopeptin synthetase has two TE domains at the C-terminal end of SypC. The first domain is 260 amino acid residues and contains the complete nucleophile elbow with G × S × G motif and conserved aspartate and histidine residues. The second TE domain, 250 amino acids, separates from the first by 11 amino acids and contains the conserved G x S x G and aspartate residues but no histidine. The TE domains are probably involved in the hydrolytic cleavage of syringopeptin and cyclisation at the C-terminal region to form a lactone ring (Brunner et al. 2002; Schwarzer et al. 2003).

11.6.2.5 Genetics and Regulation

The regulation of syringomycin and syringopeptin production is complex and is considered similar if not common. Syringopeptin production is stimulated by plant signal molecules such as phenolic glycosides (arbutin), flavonol glycosides (quercetin 3-rutinosyl-4'-glucoside and kaempferol 3 rutinosyl-4'-glucoside) and a flavanone glucoside (dihydrowogonin 7-glucoside) as well as nutritional factors particularly iron. Addition of fructose or sucrose in the presence of these factors induces a tenfold increase in transcription of *syrB-lacZ* fusion.

The regulatory gene *syrP* is located between *syrD* and *syrB* operons, codes for protein related to CheA and KinA and functions as a modulator in the multi-step phosphorelay signal transduction system.

Three regulatory genes, *salA*, *syrF* and *syrG*, lie on the right border of the syringomycin gene cluster of *P. syringae* pv. *syringae* B310D. The protein products of these genes contain C-terminal helix-turn-helix DNA-binding motif related to the luxR family of regulatory proteins. A mutation of *salA* results in non-production of syringomycin and 80 % reduction in virulence.

A functional copy of *syrF* is also necessary as mutation in *syrF* results in 88 % less inhibitory effect on *G. candidum* than the mild-type strain while mutation in the *syrG* gene resulted in 40 % reduction in virulence.

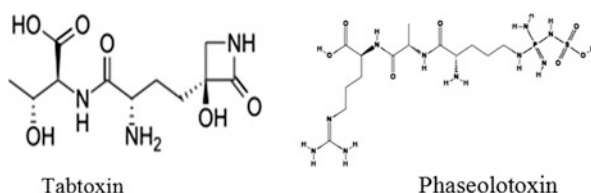
Identification of genes *salA*, *syrF* and *syrG* and the finding that *salA* is controlled by global regulators *gacS/gacA* suggests the presence of regulatory cascade controlling syringomycin production by *P. syringae* pv. *syringae* B310D. Genes *syrA* and *syrF* exhibit much greater effect on syringomycin production than syringopeptin production (Wang et al. 2006).

Secretion of syringomycin and syringopeptin is regulated by the *syrD* gene located between *syrP* and *sypA* in *P. syringae* pv. *syringae* B310D. The resulting protein *syrD* is related to proteins of the ABC transport family such as PvdE, responsible for pyoverdinin secretion in *P. aeruginosa*. ABC transporter proteins have been associated with the transport of both non-ribosomally and ribosomally encoded peptide antibiotics. The ATP-binding pocket of *SyrD* is located in the hydrophilic C-terminus and lies in the cytoplasm and the hydrophobic N-terminal

portion is inserted into the inner membrane. Mutants of *P. syringae* pv. *syringae* B310D in the *syrD* gene fail to produce both syringomycin and syringopeptin suggesting that secretion of both lipodepsipeptides is linked to *P. syringae* pv. *syringae*.

11.6.3 Antimetabolite Toxins

Some pathovars of *Pseudomonas syringae* produce small and toxic molecules called antimetabolite toxins acting as inhibitors and are named antimetabolic toxins which generally interfere with nitrogen metabolism of the host particularly as they inhibit ribosynthesis of essential amino acids resulting in an amino acid deficiency (Arrebola et al. 2011). These generally function as virulence factors and their production results in increased disease severity. They target enzymes of amino acid biosynthesis, e.g. arginine and glutamine. Common antimetabolite toxins are tabtoxin, phaseolotoxin and mangotoxin.



11.6.3.1 Tabtoxin

Tabtoxin is a dipeptide, monocyclic β -lactam antibiotic produced by *P. syringae* pvs. *tabaci*, *coronafaciens* and *garcae*. The toxin moiety, tabtoxinine- β -lactam (T β L), is linked to threonine by a peptide bond. This has been studied in *P. syringae* pv. *tabaci* BR2, a strain of phylogroup III, but has also been detected in phylogroup I and IV. Though tabtoxin is produced intracellularly, the chlorosis-inducing active moiety, T β L, is released from the native molecule by hydrolysis of a peptide bond by aminopeptidase in the plant tissue and released tabtoxinine- β -lactam (T β L) irreversibly inhibits the target enzyme glutamine synthetase (GS). This results in accumulation of ammonium causing characteristic chlorosis.

Structure T β L results in irreversible inhibition of glutamine synthetase that is responsible for two important functions: (1) as a major route for glutamine synthesis in plants and (2) efficient detoxification of ammonia (Thomas et al. 1983). Ammonia causes a number of harmful effects on plant cell including disruption of the thylakoid membrane of the chloroplast and uncoupling of photophosphorylation. The producer bacteria protect itself from the harmful effect of tabtoxin by adenylation of glutamine synthetase thus rendering the target enzyme less sensitive to T β L inactivation. The other mechanism is the production of the β -lactamase

enzyme which hydrolyses the β -lactum ring of T β L to form tabtoxinine, a nontoxic metabolite.

T β L is a chlorosis-inducing toxin and is associated with the wildfire disease of tobacco and halo blight of oats. The instability of the tabtoxin biosynthetic gene cluster results in tabtoxin-deficient variants, e.g. *P. syringae* pv. *angulata* which induces necrotic spots is considered a spontaneous toxin-deficient mutant of *P. syringae* pv. *tabaci*. The tabtoxin production genes have been reported to be clustered in a 31 kb region on the chromosome in *P. syringae* BR2 and Cit7.

When choline, betaine or dimethylglycine is used as a nitrogen source, there is at least 150 % increase in tabtoxin production by *P. syringae* strains. It has been proposed that choline is one of the plant signals that contribute towards establishment of infection by tabtoxin-producing *P. syringae* (Gallarato et al. 2012).

11.6.3.2 Phaseolotoxin

Phaseolotoxin, a nonspecific, chlorosis-inducing toxin, is produced by *P. syringae* pv. *phaseolicola* and *P. syringae* pv. *actinidiae* pathogens of legumes and kiwi fruit, respectively. Toxin produced by pv. *actinidiae* is also referred as octicidine. Phaseolotoxin comprises of an inorganic moiety, N⁸-N'-sulfodiaminophosphynil and the L-ornithyl-alanyl-homoarginine tripeptide. The octicidine lacks alanine and homoarginine residues of the native phaseolotoxin molecule.

The tripeptide may play a role in export of toxin; however, the tripeptide is not necessary for translocation/uptake into plant cells.

Phaseolotoxin acts as a competitive inhibitor of ornithine carbamoyltransferase (OCTase), an enzyme catalysing the conversion of ornithine and carbamoyl phosphate to citrulline in the urea cycle. OCTase inhibition is a reversible process and can be reversed by addition of arginine. Octicidine is more potent inhibitor of OCTase as its action cannot be reversed by arginine. OCTase inhibition results in accumulation of ornithine and deficiency of arginine leading to chlorosis (Bender et al. 1999).

Pseudomonas syringae pv. *phaseolicola* counters the toxic effect of phaseolotoxin by producing two isozymes of OCTase, one of which is resistant to toxin (ROCTase) and the other is sensitive to toxin (SOCTase). Under conditions favourable for production of toxin, i.e. temperature of 18–20 °C, ROCTase is produced. The insensitivity of ROCTase was found to be due to lower affinity for carbamoyl phosphate and slower binding to ornithine. Two independent genes have been identified to code for the two enzymes (Peet and Panopoulos 1987). Phaseolotoxin-resistant OCTase protein is a 327 amino acid polypeptide having a molecular mass of 36.52 kDa and is coded by the *argK* gene. A second mechanism of self-protection has been proposed on the basis of the production of phaseolotoxin-sensitive OCTase at 28 °C that is resistant to exogenous toxin.

A gene cluster on the chromosome is responsible for production, secretion and/or immunity. A segment pRCP17 measuring 22 kbp carries all genes involved in phaseolotoxin synthesis. Gene *argK* is also present on this segment. Zhang et al. (1993) in an independent study showed that a 25 kbp insert that complemented

with several Tox^- mutants contained eight transcriptional units. The insert was designated as pHK120 and eight transcriptional units as *phtA* through *phtH*. The pHK120 lacked *argK* but contained some sequences missing in pRCP17. The sequence analysis of a 62.6 kbp EcoR_1 fragment localised within the *phtE* locus indicated about 40 % homology to a fatty acid desaturase gene, *desA*, of other organisms. Six ORFs have been identified within *phtE* that form part of a single transcriptional unit. ORF3 encodes a protein involved in biosynthesis of ornithine, a constituent of phaseolotoxin, and has homology with acetylornithine-aminotransferase gene involved in ornithine synthesis (Zhang and Patil 1997).

A non-ribosomal, thiotemplate mechanism similar to the one used for peptide antibiotics may be involved in the synthesis of tripeptide moiety of phaseolotoxin.

The TOX^- mutants of *P. syringae* pv. *phaseolicola* were also found to be impaired in synthesis of ROCT indicating a coordinated response of the genes involved in synthesis of phaseolotoxin and *argK* coding for ROCT. Temperature plays an important role in regulation of phaseolotoxin production and infection by *P. syringae* pv. *phaseolicola*. Chlorosis of leaves of beans was induced at lower temperature (18–20 °C) and was absent at 28–32 °C. Thermoregulation of phaseolotoxin biosynthesis is mediated by a repressor synthesised at a non-permissive temperature (temperature unfavourable for phaseolotoxin synthesis).

The *argK* gene was derepressed by addition of carbamoyl phosphate to the medium even at 28 °C; however, phaseolotoxin was not detected indicating that carbamoyl phosphate affects only the *argK* gene involved in synthesis of ROCT but not the genes involved in synthesis of phaseolotoxin. This strongly suggests that (a) *argK* is under negative control of the inducer N^8 -(N' -sulfodiaminophosphinyl) group and (b) *argK* is not directly regulated by temperature but coordination with phaseolotoxin synthesis is mediated through the synthesis of the inducer which occurs at a lower temperature. It seems that more stringent dependence of phaseolotoxin synthesis on temperature may involve some other genes and proteins.

Many pathogenicity factors (and antibiotic resistance genes) that are plasmid coded are often flanked by repetitive sequences and transposable elements. These pathogenic bacteria have evolved from nonpathogenic organisms by acquiring large blocks of genetic material encoding pathogenicity and virulence rather than the slow process of adaptive evolution of pre-existing genes. A large number of essential virulence determinants such as toxins and adherence factors are found on mobile genetic elements.

In many cases, the virulence genes are clustered in large contiguous blocks found as chromosomal inserts or pathogenicity islands (Pai). These segments are acquired by illegitimate recombination resembling transposition or insertion. Pairs have been identified in many human and plant pathogens. The Pairs from *P. syringae* pv. *phaseolicola*, pv. *syringae* and pv. *tomato* and *Xanthomonas* spp. carry *vir*, *avr* and *hrp* genes. Genes for phaseolotoxin synthesis and immunity are also clustered in *P. syringae* pv. *phaseolicola* in a single segment of 270 kbp carrying *argK* and *amtA* genes. These genes must have been acquired by horizontal genetic transfer. Similar observations have been reported in *P. syringae* pv. *syringae* B301D for

syringomycin and syringopeptin synthesis. Hernandez-Guzman and Alvarez-Morales (2001) suggested that genes involved in phytotoxin synthesis in both pathovars may constitute a pathogenicity island (*Pai*).

11.6.3.3 Mangotoxin

Mangotoxin is produced by *P. syringae* pv. *syringae* isolated from mango. Mangotoxin is different from tabtoxin and phaseolotoxin the two antimetabolite toxins which are known to inhibit growth of *E. coli*. The inhibitory effect was reversed by L-ornithine and not by glutamate/glutamine and N-acetylornithine as in the case of tabtoxin and phaseolotoxin, respectively. This results in accumulation of N-acetylornithine and up to 50 % reduction in activity of ornithine N-acetyltransferase (OATase: EC2.3.1.2.5) in cell-free extracts was observed, thus interfering with ornithine/arginine biosynthesis (Fig. 11.4).

Mangotoxin is a hydrophilic oligopeptide (<3 kDa) sensitive to proteolytic enzyme but can resist extreme levels of pH and temperature. Mangotoxin affects plant metabolism, firstly as an inhibitor of arginine biosynthesis and could prevent

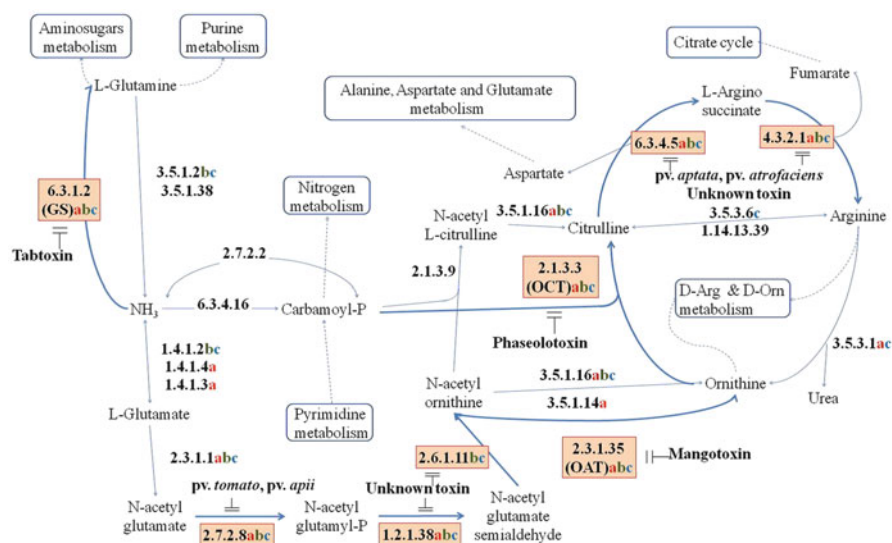


Fig. 11.4 Mode of action of different antimetabolite toxins. Enzyme code: 1.2.1.38 N-acetyl- γ -glutamyl-phosphate/N-acetyl- γ -aminoacidipyl-phosphate reductase, 1.4.1.2 glutamate dehydrogenase, 1.4.1.3 glutamate dehydrogenase NAD(P)⁺, 1.4.1.4 glutamate dehydrogenase NAD, 1.14.13.39 nitric oxide synthase, 2.1.3.3 ornithine carbamoyltransferase (OCT), 2.1.3.9 N-acetylornithine carbamoyltransferase, 2.3.1.1 amino-acid N-acetyltransferase, 2.3.1.35 glutamate/ornithine N-acetyltransferase, 2.6.1.11 acetylornithine aminotransferase, 2.7.2.2 carbamate kinase, 2.7.2.8 acetylglutamate/acetylaminoadipate kinase, 3.5.1.2 glutaminase, 3.5.1.14 aminoacylase, 3.5.1.16 acetylornithine deacetylase, 3.5.1.38 glutamine(asparagin)-ase, 3.5.3.1 arginase, 3.5.3.6 arginine deiminase, 4.3.2.1 argininosuccinate lyase, 6.3.1.2 glutamate synthase (GS), 6.3.4.5 argininosuccinate synthase, 6.3.4.16 carbamoyl-phosphate synthase (Arrebola et al. 2011/www.genome.jp/kegg/pathway.html)

protein synthesis by plant cells, secondly as an inhibitor of ornithine biosynthesis, a precursor of the polyamines, thus affecting synthesis of putrescine, spermidine and spermine essential for plant growth, especially fast-growing cells involved in processes such as flowering.

In general the antimetabolite toxins act on amino acid biosynthesis which results in amino acid deficiencies in plant tissues with simultaneous accumulation of nitrogen-containing intermediates which serve as source of nitrogen for growth of bacteria.

11.6.4 Phytohormones and Other Mechanisms

11.6.4.1 Auxin Production: IAA

A number of pathovars, viz. *Pseudomonas syringae* pv. *syringae*, pv. *aceris*, pv. *myricae* and pv. *photiniae* and *Pseudomonas savastanoi* pv. *savastanoi* produce a growth hormone, indole acetic acid (IAA), resulting in hormonal imbalance in host cells (Yamada 1993). In the presence of tryptophan, much higher amounts of IAA are produced. Their strains harbour genes homologous to *iaaM/iaaH* genes of *P. savastanoi*. For *P. savastanoi* pathogenicity implies biosynthesis of plant growth regulators as synthesis of hormones such as cytokinins and indole acetic acid leads to development of knots on olive and oleander. Infection by *P. syringae* pv. *amygdali* and *P. syringae* pv. *myricae* induces proliferation of plant tissues, and these pathovars harbour *iaaM/iaaH* genes. IAA may also act by inhibiting the host plant defence mechanism.

In *P. syringae* pv. *savastanoi* oleander strains, IAA biosynthesis from tryptophan proceeds via indole-3-acetamide (IAM). Two enzymes, a trp-2 monooxygenase and an indoleacetamide hydrolase, are involved. The genes *iaaM* (for monooxygenase) and *iaaH* (for hydrolase) are located on chromosome of *P. savastanoi* pv. *savastanoi* olive. Sequence analysis of *iaaM* and *iaaH* shows 54 % and 38 % similarity to *tms1* and *tms2*, respectively; the two genes responsible for IAA synthesis in plants are infected with *Agrobacterium tumefaciens* (Robinette and Matthysse 1990).

P. savastanoi pv. *savastanoi* oleander strains also convert IAA into indole-3-acetyl- ϵ -L-lysine (IAA-lys), which is less active than IAA. The reaction is catalysed by enzyme IAA lysine synthase encoded by the *iaaL* gene. In this strain the *iaaL* gene is located on plasmid within 2000 bp of *iaaM* and *iaaH* (Glass and Kosurge 1986). Strains related to the pathovar *savastanoi* contain high amounts of IAA. Other strains which don't harbour these genes synthesise a much less but detectable amount of IAA and arise from nonspecific nonenzymatic conversion of tryptophan into IAA. It has been suggested that even though it is established that IAA is involved in pathogenicity by *P. savastanoi* pv. *savastanoi*, other strains even though they harbour *iaaM/iaaH* genes (e.g. *Pseudomonas syringae* pvs. *syringae*, *photiniae*, *viburni* and *ribicola* and *P. savastanoi* pv. *glycinea*) don't induce proliferation of plant tissue but cause necrotic disease which is dependent upon *hrp* genes. IAA has been reported to be associated with inhibition of plant defence

mechanism as well as with epiphytic survival or with toxin production. Thus IAA production may play a role in various biological and ecological processes (Glickmann et al. 1998). In *P. syringae* pv. *tomato* DC3000, the effector protein *AvrRpt2* promotes accumulation of IAA and the increase of sensitivity to external auxin and totally modulates hormone signaling, promoting virulence (Chen et al. 2007).

11.6.4.2 Ethylene Production

Strains of *Pseudomonas syringae* pvs. *glycinea*, *pisi*, *cannabina* and *sesami* and *P. syringae* pv. *phaseolicola* isolated from kudzu (*Pueraria lobata*) produce ethylene. However, strains of *P. syringae* pv. *phaseolicola* isolated from beans don't produce ethylene. The ethylene-producing strains of *P. syringae* pv. *phaseolicola* were strongly pathogenic to kudzu, beans and soya bean as compared to the *P. syringae* pv. *phaseolicola* strains isolated from beans and not producing ethylene. Genes for ethylene production (*efe*) identified in *P. syringae* pv. *glycinea* and pv. *phaseolicola* are borne in plasmid (Volksch and Weingart 1997). Mutants disrupted in the *efe* gene in pv. *glycinea* showed reduced ability to grow in the host plant; however, *efe* mutants of pv. *phaseolicola* did not show any effect on pathogenicity (Weingart et al. 2001).

11.6.4.3 Pectinolytic Enzyme

Soft rotting bacteria, *Pseudomonas fluorescens* (*marginalis*), produce a variety of pectinolytic enzymes and degrade the pectin component of the plant-cell wall. Pectinolytic enzymes include pectin methyl esterase, pectin lyase, polygalacturonase and pectate lyase isozymes. In contrast to this, *P. viridiflava* produces a single pectate lyase (PelV) which has a very high isoelectric point like the Pel enzyme of *P. fluorescens* (*marginalis*). Sequence analysis of the *pel* gene from *P. fluorescens* and *P. viridiflava* revealed that these enzymes are members of the *Erwinia chrysanthemi* Pel ADE family (Liao et al. 1996). Mutation in the *pel V* gene revealed that this enzyme is essential for pectinolytic rot.

A number of pathovars of *Pseudomonas syringae* produce pectic enzymes, and *pel* gene sequences are available for *P. syringae* pv. *lachrymans*, pv. *phaseolicola*, pv. *tabaci* and pv. *glycinea*. *P. syringae* pv. *glycinea* produces two pectate lyase isozymes and an alkaline polygalacturonase. However, pv. *lachrymans* produces only one pectate lyase having a lower isoelectric point (8.0–8.5 pH) and is coded by the gene *pelS*.

11.6.5 Exopolysaccharide

Exopolysaccharide production has been associated with wilting by vascular pathogens and water soaking by foliar pathogens. *Pseudomonas syringae* produces two types of exopolysaccharides, the levan, a polymer of fructofuranan, and alginate, a copolymer of O-acetylated β -1,4-linked D-mannuronic acid and

L-guluronic acid (Laue et al. 2006). The gene cluster for biosynthesis of alginate by *P. syringae* pv. *syringae* is identical to *P. aeruginosa*. However, transcriptional controls and signals differ in two species. Alginate plays an important role in epiphytic fitness. The alginate-deficient mutants can form lesions but symptoms were less severe and their population was significantly reduced compared to wild type. Viscous compounds like EPS provide a means to adhere to cell surface for colonisation and biofilm formation. Mutants defective in alginate lyase (*algL*⁻) of Psy3525 were impaired in alginate biosynthesis and had reduced epiphytic fitness to cause disease symptoms (Schenk et al. 2008). Another function of EPS is suppression of induced plant defence response by calcium ion chelation (Aslam et al. 2008). Calcium ion is essential for NADPH oxidase needed for Ca²⁺-mediated defence signaling and HR cell death (Lecocourieux et al. 2006; Ma 2011). EPS also renders the cells in biofilms inaccessible to antimicrobial agents. Members of the genus *Pseudomonas* are also known to activate the multidrug efflux pump gene *mexAB/oprM* for the multidrug efflux pump. Mutants of PtoDC3000, psyB728a and Pbh1448A that are defective in the *mexAB/oprM* gene showed enhanced sensitivity to antimicrobials (Stoitsova et al. 2008).

P. syringae pv. *ciccaronei* produces mannan exopolysaccharide that has phytotoxic effects and causes chlorosis and necrosis of tobacco leaves (Corsaro et al. 2001).

11.6.5.1 Ice Nucleation

Pseudomonas syringae has been identified as an ice nucleation-active (INA⁺) bacterium that is capable of nucleating ice crystals at subfreezing temperature as high as -2 to -3°C and thereby causing frost injury to plant tissue and facilitating infection leading to foliar necrosis (Hirano and Upper 2000). This capacity is conferred by a secretory protein, the ice nucleation protein (INP). Besides *P. syringae* a number of other gram-negative phyllospheric bacteria such as *Erwinia herbicola*, *Pseudomonas fluorescens* and *Xanthomonas campestris* also show prevalence of ice nucleation protein (INP). Three genes, *inaK*, *inaV* and *inaZ*, encode three different domains of INPs (1200–1500 aas). The three structural domains of INP are (1) the N-terminal domain, about 15 % of the total length (*InaK*) which is hydrophobic in nature; (2) the C-terminal domain, approximately 4 % which is relatively hydrophilic in nature (*InaV*); and (3) central repeating domain (CRD), approximately 81 % comprising of contiguous repeats of 116 residues (*inaZ*). A gene coding for antifreeze protein has also been reported in *P. syringae* pv. *syringae* 13728a (Feil et al. 2005). Antifreeze protein is secreted into the medium and counters the growth of external ice by adsorbing onto the ice surface. Thus the modulation of ice nucleation activity by antifreeze protein protects the plant from frost injury.

Pseudomonas syringae pv. *syringae* strains, PsyB728a and PsyB310D, causing brown spots on beans and blossom blight on pears respectively exist as epiphytes and overcome the host protection system. These strains suppress the confinement mechanism of the host by production of a proteasome inhibitor, syringolin A

(sylA). This promotes infection by diffusing SylA into tissue surrounding the primary site of infection to make the tissue insensitive to immune signaling and secondly SylA promotes bacterial motility and suppresses immune response at the primary site of infection. Thus, SylA-producing bacteria are more motile and able to spread from the primary site of infection through the xylem and colonise adjacent tissues along the vascular system. Syringolin A is a small non-ribosomal cyclic peptide that irreversibly inhibits eukaryotic proteasomes (Mimas-Villamil et al. 2013).

11.7 Genomics of *Pseudomonas syringae*

Genomic analysis of a number of pathovars and strains of *Pseudomonas syringae* has been discussed in recent reviews (Dudnik and Dulder 2013; Zheng et al. 2012; Baltrus et al. 2011; Kneif et al. 2011). Diversity of *P. syringae* may be gauged by the diversity of the host plants as the strains of these have been isolated from 180 different host species across the plant kingdom and grouped into more than 50 pathovar groups. The genomes of three strains, viz. *P. syringae* pv. *syringae* strain B728a (c.o. brown spot disease of beans); pv. *tomato* strain DC3000, pathogenic to tomato and *Arabidopsis*; and pv. *phaseolicola* strain 1448A (c.o. halo blight of beans), have been completely sequenced (Feil et al. 2005; Buell et al. 2003; Joardar et al. 2005). Genomic studies provide an insight into virulence and adaptation strategies for pathogenic strains (Nguyen et al. 2010).

The genome of *P. syringae* pv. *syringae* strain B64 isolated from wheat contains the complete set of type III secretion system and ten effector proteins, AvrE1, HopAA1, HopI1, HopM1, HopAH1, HopAG1, HopAI1, HopAZ1, HopBA1 and HopZ3. Among these AvrE1, HopAA1, HopI1, HopM1 and HopAH1 constitute the effector core and are present in all the strains of *P. syringae* sequenced till date. The other five could be the host determinants for wheat. In addition there are two type VI secretion system gene clusters and nine putative effector proteins belonging to *VgrG* and *Hep1* families. Besides these four phytotoxins, syringomycin, syringopeptin, syringolin and mangotoxin, are also coded by the genome of *P. syringae* pv. *syringae* strain B64.

Additional traits, exopolysaccharide alginate, polysaccharide synthesis locus (Ps1) and levan biosynthesis, surfactin syringofactin, type VI pili, large surface adhesions, siderophores pyoverdine and achromobactin, proteases and other secreted hydrolytic enzymes and RND-type transporters that are associated with virulence have been identified in PssB64. The ice nucleation gene *inaZ* is truncated and thus the strain lacks ability of ice nucleation.

Studies on population structure and dynamics of the core genome of *P. syringae* were undertaken by multilocus sequence typing of 80 strains representing 21 pathovars and two nonpathogens (Sarkar and Guttman 2004). Seven housekeeping genes coding for sigma factor 70 (*rpoD*), gyrase (*gyrB*), aconitate hydratase B (*achB*), citrate synthase (*cts*), glyceraldehyde-3-phosphate

dehydrogenase (*gap*), phosphoglucosomerase (*pgi*) and phosphofructokinase (*pfk*) were sequenced. Forty unique types were identified with most of the strains falling into one of the four major clades. Phylogenetic analyses indicated a common evolutionary history of the seven loci with very low level of recombination. Analysis further supports that *P. syringae* is a highly clonal organism. Mutation is more likely to change a nucleotide than recombination.

If the core genome of *P. syringae* were responsible for determining host specificity, it would mean that variation in housekeeping genes would be tightly associated with the host plant and the factor outside the core genome must be maintaining the cohesion of the species and play a significant role in determining the host suitability. Thus the flexible genome markers, viz. virulence factors such as type III effector proteins, phytotoxins and resistance genes, play a role in determining the host suitability.

TTSS Effector Proteins, Helpers and Other Members of the *HrpL* Regulon TTSS is an essential mediator for interaction of a phytopathogen with the plant host and shares a common ancestor with bacterial flagellum. TTSS translocates an array of proteins, the type III effector proteins through type III pilus to extracellular location or to plant cell.

Hop effector proteins play a key role as virulence factors for effective suppression of the host resistance mechanism and effective infection following entry into plant tissue. All 46 families of Hop effectors and seven families of helper proteins have been identified on the basis of their regulation by HrpL alternate σ -factor and translocation by TTSS.

Plant target sites and specific activities of effectors AvrPt01, AvrPt0B, AvrE1, HopF2, HopI1, HopU1, HopM1, HopN1, HopQ1-1 and HopA01 have been established. HopAK1, HrpZ and HrpW play an essential role in translocation of effector proteins in plant cell and have properties similar to harpins. The helper proteins *HrpH*, *HopAJ1* and *HopP1* also have a lytic transglycosylase activity and facilitate translocation type III pilus components through the peptidoglycan layer (Tampakaki et al. 2010).

Another type of pathogenicity factor referred as type VI secretion system (T6SS) has also been discussed in *Pseudomonas cannabina* pv. *alisalensis* (*Pcal*) (Sarris et al. 2010, 2013). It is widespread and occurs in multiple copies in the genome of phytopathogens. T6SS is considered to promote commensal or mutualistic relationship between bacteria and eukaryotes and mediate cooperative and competitive interactions between bacterial species.

Five strains of *Pcal* examined showed similarity in loci involved in biogenesis of type I, II, III and VI secretion systems. Besides these genes they also possess genes for the AT-1 family of type V secretion system, general secretion (*sec*) pathway and twin-arginine translocation (*Tat*) pathway. All these systems have also been found in other members of plant-pathogenic pseudomonads except that T1SS is absent in the genome of PstDC3000 (Cunnac et al. 2009). Complete *hrp/hrc* gene clusters

encoding TTSS of the 'HrpI family' were found in all members of *Pcal* and closely resembled *P. syringae* B728a. *Pcal* strains also contain two clusters for T6SS and the exact role for the gene products has not yet been established.

The *hrp/hrc* locus spans 25658 bp in *Pcal* and is composed of 28 genes arranged in five operons organised in two blocks having convergent transcription: the *hrpRS*, *hrpZ* and *hrpC* being transcribed in one direction and *hrpU* and *hrpJ* being transcribed in the opposite direction, the two blocks are separated by a hyper-variable region that harbours two ORFs. This is similar to the one reported for *P. syringae* pathovars.

Distribution of Type III Effector Genes in *Pcal* Strains Analysis of the Hop database (HDB: <http://www.pseudomonassyringe.org>) for nucleotide and amino acid sequences showed that core T3EPs (Type III effector proteins) are conserved in all five strains of *Pseudomonas cannabina* pv. *alisalensis* (*Pcal*) as well as strain-specific effectors.

1. A subset of 19 genes forms the core TTSS repertoire of *Pcal*: *avrE1*, *avrPt05*, *hopAA1a*, *hopAA1b*, *hopAB3-1*, *hopR1*, *hopV1*, *hopW1-1*, *hopW1-2*, *hopX1a*, *hopAF1*, *hopAL1*, *hopA01*, *hopAQ*, *hopAS1*, *hopD1*, *hopI1*, *hopM1*. They play an important role in pathogenicity of *Pcal* and that is the reason for their conservation.
2. The second subset of genes comprises of *avrRpm1*, *hopAB3-2*, *hopAC1*, *hopAD*, *hopAE1*, *hopAH1*, *hopQ1*, *hopX2* and *hopAD1*.
3. The third subset of gene coding of TTSS was strain specific: *avrPt01*, *hopAM1*, *hopAT1*, *hopAU1*, *hopAV1*, *hopAW1*, *hopAY1*, *hopAZ1*, *hopBD1*, *hopBD2*, *hopBF1*, *hopBG*, *hopE1*, *hopG*, *hopO1*, *hopT1*, *hopX1b* and *hopZ1*.
4. This subset comprises of truncated strain-specific effectors: *hopAG1*, *hopAR1*, *hopBB1* and *hopH1*.

Two genomic clusters in *Pcal* have been identified for T6SSs (T6SSI and T6SSII). T6SSI comprises of 16 conserved genes and is closely related to *P. aeruginosa* T6SSI; T6SSII is very similar to T6SSII cluster of Pst DC3000 except that *Pcal* lacked regulatory genes *ppKA* and *pppA*.

Phytotoxins play an important role in pathogenicity of *Pseudomonas* plant pathogens, and a number of genes involved in synthesis and regulation of phytotoxins have been identified.

Pseudomonas cannabina pv. *alisalensis* (*Pcal*) genes and gene clusters showed high level of similarity to Pst DC3000 coronamic acid synthases: 100 % identity with *CmaD*, 99 % identity with *CmaE*, 93 % identity with *CmaA*, 100 % identity with *CmaB*, 100 % identity with *CmaC* and 99 % identity with *CmaT*. The IAA lysine synthetase (*iaaL*) showed 96 % identity with *iaaL* of Pst DC3000 and 92 % identity was observed in tabtoxin resistance protein of *P. syringae* pv. *tabaci*.

11.7.1 Plasmids of *P. syringae* and Pathogenicity

Members of the genus *Pseudomonas* harbour a wide array of plasmids coding for degradation of complex and chlorinated organic compounds, resistance to antibiotics and promoting plant pathogenicity. Besides this the mobile elements associated with plasmids and chromosomes play an important role in acquisition of determinants that code for virulence and geological fitness that enhance adaptation. Many of these are acquired through horizontal transfer from other bacteria. Thus plasmids may be responsible for the phenotypes like pathogenicity, host specificity, toxin, hormone production and resistance to bactericides such as antibiotics.

Genes involved in pathogenicity and host specificity comprise two main groups—avirulence (*avr*) and virulence (*vir*) genes (Vivian and Gibbon 1997) and those involved in the type III protein secretion system, the ‘harp’ (*hrc/hrp*) genes (Galan and Collmer 1999). TTSSs are generally located on chromosomes and are also present in animal pathogens. A number of plasmid genes involved in pathogenicity and host specificity are listed in Table 11.4. (Zhu et al. 1995; Vivian et al. 2001).

The cluster of genes involved in TTSS are borne on chromosomes in *P. syringae*, *X. campestris* and *E. amylovora*; however, in *Erwinia herbicola* pv. *gypsophila*, the *hrp* genes are on the 150 kb plasmid pPATH (Nizan et al. 1997) and in *R. solanacearum* these genes are on a mega-plasmid, pVIR. The plasmid-borne counterparts are similar to their chromosomal homologues.

The TTSSs serve to deliver effector proteins into plant cells including *avr* and *vir* gene products. The virulence gene products are extracellular enzymes, plant hormones and toxins while no specific role had been assigned to *avr* gene products and are evenly divided between plasmids and chromosomes (Vivian and Gibbon 1997). Avirulence genes have been identified to an HR in plant host and carry a matching gene for resistance (R) as per the gene per gene theory. Six pairs of *avr*/R genes have been recognised. Their presence in the pathogen and host results in HR, while absence of this matching leads to development to disease (Taylor et al. 1996).

Eight of the nine races of *P. savastanoi* pv. *phaseolicola* causing halo blight of beans harbour large plasmid of ~150 kb. A strain cured of plasmid, pAV511, resulted in loss of virulence but continued to elicit HR indicating that chromosomal genes are responsible for elicitation of a defence reaction. A low G + C ratio region of a 30 kb pathogenicity island (*Pai*) was identified to be responsible for virulence. Introduction of the *vir* *pphA* gene into cured strain RW60 restored virulence to beans while maintaining avirulence to soya bean (*Glycine max*). Curing of an 82 kb native plasmid in *Ps. syringae* pv. *eriobotryae* (c.o. stem canker of loquat) resulted in loss of pathogenicity and a single gene, *psvA*, was capable of restoring pathogenicity. Like *vir* *pphA*, *psvA* is flanked with similarity to transposase and some noncoding regions.

Genes coding for coronatine that is responsible for chlorosis and stunting are generally borne on plasmids; however, in pv. *maculicola* genes coding for coronatine are borne on chromosome. In *P. savastanoi* pv. *glycinea* and *P. syringae* pv. *tomato*, coronatine production is coded by a 30 kb *cor* gene cluster

Table 11.4 Plasmid-borne pathogenicity character in *Pseudomonas syringae* and related species [Modified Vivian et al. (2001)]

S. No.	Character	Plasmid name	Size (kb)	Strain/pathovars
1	Coronatine	pMAC1	83	<i>Psy</i> pv. <i>maculicola</i> H3-6
		ND	105	<i>Psy</i> pv. <i>morsprunorum</i>
		p4180A	90	<i>Psa</i> pv. <i>glycinea</i> st. P94180
		pCOR1	88	<i>Psy</i> pv. <i>atropurpurea</i>
		pPT23A		<i>Psy</i> pv. <i>tomato</i> PT23.2
2	Cytokinin	pCK1	42	<i>Psa</i> pv. <i>savastanoi</i> Ps93
		pCK1	105	<i>Psa</i> pv. <i>savastanoi</i> EW1006
3	Pathogenicity/vir/avir gene	pAV505	140	<i>Psa</i> pv. <i>savastanoi</i> 1302A
		pAV511	154	<i>Psa</i> pv. <i>savastanoi</i> 1449B
		ND	82	<i>Psy</i> pv. <i>erobotryae</i> NAE 6
	vir gene	pDC3000A	64	<i>Psy</i> pv. <i>tomato</i> DC3000
4	Resistance to antibiotics, heavy metals, UV, etc.			
	strA, strB, conjugative smr	pCPP519	83	<i>Pseudomonas</i> spp. st. PyR19
		pCPP501	108	<i>Psy</i> pv. <i>papulans</i> Psp36
		pCPP511	89	<i>Psy</i> pv. <i>papulans</i> Psp47
	Cu ^r , Co ^r , As ^r , mucoid	pPSR12	200	<i>Psy</i> pv. <i>syringae</i> B3010
		pPSR1	68	<i>Psy</i> pv. <i>syringae</i> A2
5	Hormones			
	Ethylene	pPSP1	68	<i>Psa</i> pv. <i>phaseolicola</i> Ludzu Pk2
		pETH1	110	<i>Psy</i> pv. <i>cannabina</i> MAFF 302256
	Indole acetic acid	pIAA1	52	<i>Psa</i> pv. <i>savastanoi</i> EW2009
		pIAA2	72	<i>Psa</i> pv. <i>savastanoi</i> PB213
6	Effector gene			
	avr PphD	pAV505	140	<i>Psa</i> pv. <i>phaseolicola</i>
	avr PphF, R1, avr PphC, avrD5, avr PphA	pAV511	154	<i>Psa</i> pv. <i>phaseolicola</i>
	avrC, RPG3 (soya bean)	ND	150–200	<i>Psa</i> pv. <i>glycinea</i>
	psv A (loquat)	ND	82	<i>Psy</i> pv. <i>erobotryae</i>
	avr D3 (soya bean)	ND	90	<i>Psy</i> pv. <i>lachrymans</i>
	avr D4 (soya bean)	ND	75	<i>Psy</i> pv. <i>lachrymans</i>
	avr Ppi A2.R2	pAV3282	47	<i>Psy</i> pv. <i>pisi</i> Race 7
	avr Ppi A3.R2	pAV3282	45	<i>Psy</i> pv. <i>pisi</i> Race 7
	avr Ppi B1.R3	pAV3282	40	<i>Psy</i> pv. <i>pisi</i> Race 7
	avr Ppi B2.R3	pAV3282	66	<i>Psy</i> pv. <i>pisi</i> Race 7
	avr Ppi B3.R3	pAV3282	66	<i>Psy</i> pv. <i>pisi</i> Race 7
	avr Ppi G	pAV3282	45	<i>Psy</i> pv. <i>pisi</i> Race 7
	avr D1.RPG4	pAV3282	83	<i>Psy</i> pv. <i>tomato</i>
7	Cryptic plasmids (loss does not affect pathogenicity)	pBPW1	48	<i>Psy</i> pv. <i>tobaci</i> BR2
		pOSU900	80	<i>Psy</i> pv. <i>syringae</i> J900
		ND	94.5	<i>Psy</i> pv. <i>syringae</i> NAE6

borne on a 90–100 kb plasmid (Bender et al. 1996). The plasmid-borne *cor* genes are always associated with sorbitol utilisation (Cuppels and Ainsworth 1995). The coronafacic acid gene cluster in *P. syringae* pv. *tomato* PT23 is located on pPT23A and is required for expression of virulence determinants on a closely related plasmid, pPT23B (Sesma et al. 2001).

Even for production of plant hormones, IAA, the specific trait is borne on plasmid in *P. savastanoi* pv. *savastanoi* infecting oleander (*Nerium oleander*), while it is chromosomally borne in strains infecting olive (*Olea europaea*) and ash (*Fraxinus excelsior*). As such the strains appear to be host specific. Oleander and olive strains also produce another hormone, cytokinin, specified by the *ipt* (*ptz*) gene in *P. savastanoi* pv. *savastanoi*. High levels of IAA are produced in the presence of tryptophan. This has been correlated to the presence of homologues *iaaM* and *iaaH* in *P. savastanoi* pv. *savastanoi*; in pvs. *myricae* and *photiniae*, these genes are located on plasmids. IAA is possibly produced by an alternate pathway involving indole pyruvate (Glickmann et al. 1998). The ethylene production gene *efe* is also borne on plasmid pPSP1 in *P. savastanoi* pv. *phaseolicola*, *P. syringae* pv. *cannabina* and *P. savastanoi* pv. *glycinea*. Plasmids have also been identified for resistance to copper ion and exposure to UV radiation and to antibiotics, e.g. streptomycin. Copper resistance is often linked to streptomycin resistance and the genes are borne on a conjugative plasmid in *P. syringae* pv. *syringae* ranging in the size of 68–220 kb (Sundin and Bender 1993). It also carries *Sm*[®] transposon Tn5393. A number of *Is* elements are present in the plasmid DNA and depending on their location inactivate a gene and also serve as site for homologous recombination. *Is51* and *Is52* insert in *iaaM* gene in *P. savastanoi* pv. *savastanoi* resulting in loss of ability to induce gall formation.

Plasmid pPT23A Family Many *P. syringae* strains have been reported to contain plasmids collectively referred as pPT23A family that cross-hybridise with replication sequences from pPT23A of *P. syringae* pv. *tomato* PT23. These plasmids encode determinants that are important for host–pathogen interactions such as coronatine-biosynthesis locus which increases virulence and host range, plasmid stability locus (*stb* CBAD), copper resistance and *snR* transposon *Tn5393* and *Is* elements *Is51*, *Is801*, *Is870* and *Is1240*. Comparative genomic analysis of 31 pPT23A family plasmids of *P. syringae* indicated that *repA* is the defining parameter of this family. Phylogenetic analysis did not show any relationship with their hosts indicating that horizontal gene transfer and recombination have contributed to evolution of pPT23A family plasmids (Zhao et al. 2003).

11.7.2 Biotechnology for Novel Bioactive Compounds and Transgenic Plants

Pseudomonas syringae strains producing phytotoxins show resistance to the phytotoxin molecule. One such gene, *argK*, for resistant ornithine carbamoyl-transferase (ROCTase) has been identified for resistance to phaseolotoxin.

A similar gene for resistance to tabtoxin (*ttr*) has been isolated from *P. syringae* (Anzai et al. 1989). The specific genes have been cloned in plants for countering the effect of the phytotoxin produced by an infecting organism.

Molecular techniques may also be used to generate peptides and polyketides with altered biological properties to design bioactive peptides and herbicides (Nagraj and Singh 2010; Win et al. 2012). Molecular biology of microbe–plant interactions has brought forward the central role for plant pathogen effector molecules and has resulted in emergence of new concepts in phytopathology. However, yet much is not known about the identity and function of effectors of many pathogens including the obligatory plant pathogens. Comparative genomics of *Pseudomonas syringae* is providing useful information for developing strategies for ecofriendly and effective control of phytopathogens.

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